UAlg FCT
UNIVERSIDADE DO ALGARVE
FACULDADE DE CIÊNCIAS E TECNOLOGIA

Antioxidant and antidiabetic properties of medicinal plant infusions

Daniela Pedro Lourenço

Dissertation to obtain the Masters degree in
Mestrado Integrado em Engenharia Biológica

2016

UNIVERSIDADE DO ALGARVE
FACULDADE DE CIÊNCIAS E TECNOLOGIA

Antioxidant and antidiabetic properties of medicinal plant infusions

Daniela Pedro Lourenço

Dissertation to obtain the Masters degree in
Mestrado Integrado em Engenharia Biológica

Realized under the orientation of:
Professora Doutora Isabel Saraiva de Carvalho

Faro, Setembro de 2016

Antioxidant and antidiabetic properties of medicinal plant infusions

Declaração de autoria de trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

_____

Daniela Pedro Lourenço

Agradecimentos

Agradeço do fundo do meu coração aos meus pais e namorado pelos anos de apoio e incentivo que me deram em prol de um futuro melhor. Pela paciência de muitas frustrações e lágrimas e por todo o esforço que nem sempre foi recompensado.

À professora Doutora Isabel Carvalho por toda a dedicação, paciência e simpatia demonstrada. Um muito obrigado por me ter recebido no seu laboratório, pelo excelente espírito de convívio que lá existe e pelas saídas de campo.

Ao brevemente Doutor Ricardo Nunes pelas incontáveis horas no laboratório a trabalhar e a ajudar a trabalhar por quem lá passou. Agradeço toda a paciência e espírito de companheirismo demonstrado e acima de tudo um amigo que ganhei.

À Doutora Ana Anastácio pelo espírito crítico e construtivo de ver as coisas, pelas boas ideias e opiniões e principalmente pela forma "despachada" de ser mas que faz as coisas andarem para a frente.

Ao Doutor Vahid e ao Doutor Raja pela sincera disponibilidade prestada ao longo destes meses e momentos de convívio que ficam na lembrança.

Ao Coronel José Rosa Pinto e a todos aqueles que nos acompanham e auxiliam diariamente ou esporadicamente, direta ou indiretamente mas que contribuíram para a realização deste projeto de vida.

Sejam quais forem os resultados, com êxito ou não, o importante é que no final cada um possa dizer: 'fiz o que pude'.

Louis Pasteur (1822 - 1895)

Resumo

Este trabalho teve como objetivo principal a avaliação das atividades antioxidantes e antidiabéticas de sete plantas do Algarve. Para tal foram usadas infusões aquosas a quatro temperaturas diferentes (25, 50, 75 e 95°C), de forma a determinar qual a melhor planta e qual a melhor temperatura.

As plantas testadas, selecionadas pela sua relevância na medicina tradicional e usadas comumente pelas populações, foram a *Centaurium erythraea* Rafn. (CE) ou fel-da-terra, a *Cistus ladanifer* L. (CL) ou esteva, a *Myrtus communis* L. (MC) ou murta, a *Rosmarinus officinalis* L. (RO) ou alecrim, e três tipos de tomilhos: *Thymbra capitata* (L.) Cav. (TC), *Thymus albicans* Hoffmanns & Link (TA) e o *Thymus lotocephalus* G. López & R. Morales (TL).

As infusões foram feitas usando as partes aéreas das plantas secas (inicialmente reduzidas a um pó grosseiro). Destas 0,5 g foram adicionadas a 20 mL de água, previamente aquecida em banho-maria à temperatura desejada. Após 15 min (sem agitação) as infusões foram filtradas e devidamente armazenadas a - 20°C no congelador até à realização dos testes.

No teor em conteúdo de fenólicos totais a infusão MC apresentou de um modo geral os valores mais elevados para as quatro temperaturas testadas (25, 50, 75 e 95°C) variando entre os valores médios de 356 e 383 mg GAE/g dw. A infusão CE por sua vez apresentou valores inferiores variando entre 43 e 50 mg GAE/g dw. No conteúdo de flavonoides totais a infusão CL obteve valores superiores às restantes plantas ao variar entre os 46 e 29 mg QE/g dw enquato na TC foram obtidos os valores inferiores, entre os 5 e os 8 mg QE/g dw. Na atividade antioxidante total ambas as infusões de CL e MC voltaram a apresentar os maiores valores e muitos próximos entre si, variando entre 404 e 615 mg AAE/g dw para a CL e entre 465 e 531 mg AAE/g dw para a MC. Os menores valores obtidos foram os da infusão CE que variaram entre 216 e 255 mg AAE/g dw.

Quanto aos métodos redutores avaliados, RP e FRAP, a infusão MC demonstrou, mais uma vez, ter maior atividade do que as restantes variando entre 200 e 317 e 848 e 943 mg TE/g dw, respectivamente. Novamente a infusão de CE apresentou os valores inferiores em ambos os métodos variando entre 28 e 35 e 53 e 72 mg TE/g dw para o RP e FRAP, respetivamente.

Relativamente à captura de radicais livres, nomeadamente no DPPH e no ABTS, a infusão MC foi uma vez mais a que apresentou melhores resultados, ou seja, valores de IC_{50}

(concentração de extrato para a qual 50% dos radicais livres são capturados) mais baixos variando entre os 11 e os 15 µg/mL, enquanto a CE foi novamente a planta com os piores resultados, e neste caso com o IC_{50} mais elevado variando entre os 331 e os 446 µg/mL. Todas as infusões apresentaram valores menores de IC_{50} no método do DPPH quando comparados com os valores obtidos pelo método do ABTS.

Foi seguidamente quantificada a proporção de amarelos, vermelhos e azuis das infusões obtidas a 25 e a 75°C, através de um método de análise de cor, sendo o amarelo a cor em maior proporção. Em simultâneo a atividade antidiabética foi avaliada pelo método da α-amilase sendo possível obter resultados nas infusões de MC, CL e CE. Para tal foi usada uma reta de calibração com acarbose e para a temperatura de 25ºC. A infusão CE apresentou o valor de 192, a CL obteve 674 e a MC obteve um valor de 2004 µg AcE/g dw. A 75ºC todas as infusões exceto a CE e a TC apresentaram actividade antidiabética, sendo que a CL obteve o maior valor com 946 µg AcE/g dw e a TA o menor valor com 154 µg AcE/g dw. O método da α-glucosidase foi testado para as infusões a 25ºC e mais uma vez a CL e MC foram as que apresentaram melhores valores (acima de 4000 mg AcE/g dw) e CE foi novamente a que obteve um valor inferior de 16 mg AcE/g dw.

De toda a avaliação realizada neste estudo de um modo geral os melhores resultados foram obtidos nas infusões feitas a 75°C e, por outro lado, a 50°C os piores. As infusões obtidas a partir das plantas RO, TC, TA e TL (as aromáticas) apresentaram um comportamento semelhante em todos os métodos.

Verificou-se também que as plantas aromáticas (RO, TC, TA e TL) foram ao longo dos métodos realizados as que sofreram uma maior variação de valores em cada temperatura de infusão, sugerindo que estas são mais influenciadas pela temperatura do que as restantes. O oposto foi verificado para as infusões de CE, CL e MC, sugerindo que mesmo variando a temperatura de extração, os compostos bioativos presentes prevalecem.

Por fim, com o auxílio do programa SPSS® 22 foi possível fazer as correlações entre os diferentes métodos e verificou-se que as correlações eram significativas entre todos eles. Apesar de serem significativas, quando se correlaciona o TFC com os restantes métodos estas são menores e as melhores são do RP com o TPC e FRAP.

Fizeram-se ainda três dendrogramas a partir dos quais é possível verificar as diferenças entre os métodos testados e as infusões. Para os métodos foram identificados três *clusters*, um com o TFC, outro com o TAA e os restantes constituem o terceiro *cluster*. Relativamente à análise por plantas outros três *clusters* foram identificados, um correspondente às infusões das plantas aromáticas, outro para as infusões MC e CL e finalmente a CE. No terceiro

dendrograma são apresentadas todas as infusões a cada temperatura de extração e cinco *clusters* foram identificados, três deles com uma única infusão cada (CE, MC e CL), os outros dois correspondem, no geral, às infusões das plantas aromáticas mas agrupadas por temperaturas (25 e 50°C; 75 e 95°C).

É possível concluir que, ao contrário da fel-da-terra (CE) que apresentou os piores resultados, plantas como a murta (MC) e a esteva (CL) têm um forte potencial antioxidante e antidiabético (ainda subaproveitado), essenciais para a prevenção e tratamento de muitas doenças inflamatórias, degenerativas e vários distúrbios, resultantes em grande parte do stress oxidativo.

Termos chave: plantas medicinais; atividade antioxidante; atividade antidiabética; compostos fitoquímicos.

Abstract

The main goal of this dissertation was to evaluate the antioxidant and antidiabetic activities of infusions at four temperatures (25, 50, 75 and 95ºC) from seven plants of Algarve.

The tested plants, used in traditional medicine, are the *Centaurium erythraea* Rafn. or small centaury, *Cistus ladanifer* L. or gum rockrose, *Myrtus communis* L. or myrtle, *Rosmarinus officinalis* L. or rosemary, and three types of thyme: *Thymbra capitata* L. Cav., *Thymus albicans* Hoffmanns. & Link and *Thymus lotocephalus* G. López & R. Morales.

The infusions, were made using half gram (of the dried aerial parts) in 20 mL of distilled water for 15 min, previously heated at the desired temperature, filtered and stored at -20ºC.

Myrtus infusion had the best results and *Centaurium* the worst in the antioxidant methods (TPC, TAA, RP, FRAP, DPPH and ABTS) except TFC for which *Cistus* and *Thymbra* had the best and worst respectively. In α-amylase assay, *Myrtus* and *Cistus* infusions were the best at 25 and 75ºC respectively and the same was observed in α-glucosidase assay at 25ºC.

In general the best results were obtained for the temperature of 75ºC and the worst for 50ºC.

Analysing the correlations, TFC had the worst correlations with the other assays and RP the best ones. In the dendrograms referring to the methods, plants and plant at each temperature, 3 clusters were identified in the first two and 5 clusters in the last one.

Myrtus and *Cistus* have a strong antioxidant and antidiabetic activity, essential to prevent or treat some diseases, including those caused by oxidative stress. Due to the rich content in biological active compounds it is possible to use them in a preventive and balanced diet.

Keywords: medicinal plants; antioxidant activity; antidiabetic activity; phytochemical compounds.

Table of contents

1. General introduction……1
1.1. Medicinal plants……..2
1.1.1. *Centaurium erythraea* Rafn. ……..4
1.1.2. *Cistus ladanifer* L. ..……...…….5
1.1.3. *Myrtus communis* L. …...……7
1.1.4. *Rosmarinus officinalis* L. ……...8
1.1.5. *Thymbra capitata* L. Cav., *Thymus albicans* Hoffmanns. & Link, *Thymus lotocephalus* G. López & R. Morales ……..9
1.2. Antioxidants – reactive oxygen species and consequences ……11
1.3. Diabetes mellitus ……..12
2. Objectives ……13
3. Material and Methods ……...15
3.1. Chemicals ……16
3.2. Equipment ……16
3.3. Plant material ……...16
3.4. Methodology ……17
3.4.1. Extraction process ……17
3.4.2. Total phenolic content (TPC) ……...17
3.4.3. Total flavonoid content (TFC) …….....17
3.4.4. Total antioxidant activity (TAA) …….....18
3.4.5. Reducing power (RP) …….....18
3.4.6. Ferric reducing antioxidant power (FRAP) ……..18
3.4.7. DPPH free radical scavenging activity (DPPH) ……...19
3.4.8. ABTS free radical scavenging activity (ABTS) ……...19
3.4.9. Colourimetric analysis …….....19
3.4.10. α-amylase inhibition assay (α-amylase) ……...20
3.4.11. α-glucosidase inhibition assay (α-glucosidase) ……....20
3.4.12. Statistical analysis ……....21
4. Results and discussion ……..22
4.1. Phenolic compounds and antioxidant activity ……..23
4.1.1. Total phenolic content and total flavonoid content ……..23
4.1.2. Total antioxidant activity, reducing power and ferric reducing antioxidant power ……27

4.1.3. DPPH free radical scavenging activity and ABTS free radical scavenging activity ……..……30
4.1.4. Correlations and dendrograms ……..34
4.1.5. General conclusions ……38
4.2. Colourimetric analysis and antidiabetic activity ……..39
4.2.1. Colourimetric assay ……39
4.2.2. Antidiabetic activity ……41
4.2.3. General conclusions ……....43
5. Final conclusion ……45
6. Bibliographic references……....47

List of figures

Figure 1. Algarve map with the seven studied plants collection sites. ……2
Figure 2. *Centaurium erythraea* Rafn. plant. ……....4
Figure 3. *Cistus ladanifer* L. plant. ……...5
Figure 4. *Myrtus communis* L. plant. ……....7
Figure 5. *Rosmarinus officinalis* L. plant. …….……....8
Figure 6. *Thymbra capitata* L. Cav., *Thymus albicans* Hoffmanns. & Link *and Thymus lotocephalus* G. López & R. Morales plants. ……....9
Figure 7. Dendrograms of the global relations between assays (A), plants (B) and plants at each temperature (C). ……...…....36
Figure 8. Colour of the seven plant infusions (diluted to 0.003 g/mL) obtained at 25 and 75ºC, respectively. ……...……....39

List of Tables

Table I. Scientific and common names, code, voucher number and medicinal uses and properties of the seven plants studied. …….....4
Table II. TPC and TFC of the seven plant infusions obtained at four different temperatures.24
Table III. Graphical representation of temperature influence in TPC (mg GAE/g dw) and TFC (mg QE/g dw) results. Values obtained in each assay (y axis) at 25, 50, 75 and 95°C (x axis). Different letters in each graph correspond to statistically different results ($p<0.05$). 25
Table IV. TAA, RP and FRAP of the seven plant infusions obtained at four different temperatures. …….....28
Table V. Graphical representation of the means with standard deviations obtained from three replicates in TAA (mg AAE/g dw), RP (mg TE/g dw) and FRAP (mg TE/g dw) assays (y axis) at 25, 50, 75 and 95°C (x axis). …….....29
Table VI. DPPH and ABTS radical scavenging activities IC50 of the seven plant infusions obtained at four different temperatures. …….....32
Table VII. Graphical representation of the means with standard deviations of the IC50 values obtained from three replicates in DPPH (μg/mL) and ABTS (μg/mL) assays (y axis) at 25, 50, 75 and 95°C (x axis). …….....33
Table VIII. Pearson's correlations (*r*) of the antioxidant assays (infusions obtained at all temperatures). ……...……....34
Table IX. Colour of the infusions obtained at 25 and 75ºC. ……40
Table X. Results of the antidiabetic activity of the seven plant infusions in acarbose equivalents determined by α-amylase (infusions at 25 and 75ºC) and α-glucosidase (infusion at 25ºC). ……...…….……....42

Abbreviations, acronyms and symbols

AAE	Ascorbic acid equivalents
Abs	Absorbance
ABTS	2–2'–azinobis–(3–ethylbenzothiazoline–6–sulfonic acid)
AcE	Acarbose equivalents
Cav.	Cavanilhes (António José)
CE	*Centaurium erythraea* L.
CL	*Cistus ladanifer* L.
DPPH	2,2–diphenyl–1–picrylhydrazyl
dw	Dry weight
FRAP	Ferric reducing antioxidant power
g	Gram
GAE	Gallic acid equivalents
HCA	Hierarchical cluster analysis
IC_{50}	Inhibitory concentration
iABTS	Inverse value of ABTS free radical scavenging activity IC_{50}
iDPPH	Inverse value of DPPH free radical scavenging activity IC_{50}
L.	Lineus
M	Molar
MC	*Myrtus communis* L.
mg	Milligrams
min	Minutes
mL	Milliliters
mM	Millimolar
nm	Nanometer
n/d	Not determinable
pNGP	4-Nitrophenyl-α-D-glucopyranoside
QE	Quercetin equivalents
RO	*Rosmarinus officinalis* L.
RP	Reducing power
T	Temperature
TA	*Thymus albicans* Hoffmanns. & Link

TAA	Total antioxidant activity
TC	*Thymbra capitata* L. Cav.
TE	Trolox equivalents
TFC	Total flavonoid content
TL	*Thymus lotocephalus* G. López & R. Morales
TPC	Total phenolic content
TPTZ	2,4,6–tripyridyl–2–triazine
U/mL	Unit/milliliter
UV/Vis	Ultraviolet/visible
w/v	Weight/volume
µg	Micrograms
ºC	Celsius degrees
%	Percentage
% I	Inhibitory percentage

1. General introduction

1.1. Medicinal plants

Since the earlier times that human being as a recolector, search and collect many plant species, wich were use for food purposes or to treat ailments and diseases. Through generations, this knowledge about the uses of certain plant species to treat several problems prevailed in traditional medicine, since until a few decades ago the access to medicines was difficult and too expensive.

Mediterranean area has a temperate climate wich is recognize by the abundant variety of plants, many with well known properties which are used in traditional medicine. These medicinal and aromatic plants have chemical compounds with an important biological function in their own defenses. Portugal and especially the Algarve have these Mediterranean characteristics and many autochthonous plants showed over the years that have excellent properties to treat several ailments.

Seven plants were collected from three diferent parts of Algarve (Figure 1). The rockrose was harvested in the west zone of Algarve in Lagos, small centaury in Loulé and in Gambelas campus from University of Algarve the myrtle, rosemary, mediterranean thyme and more two types of thyme.

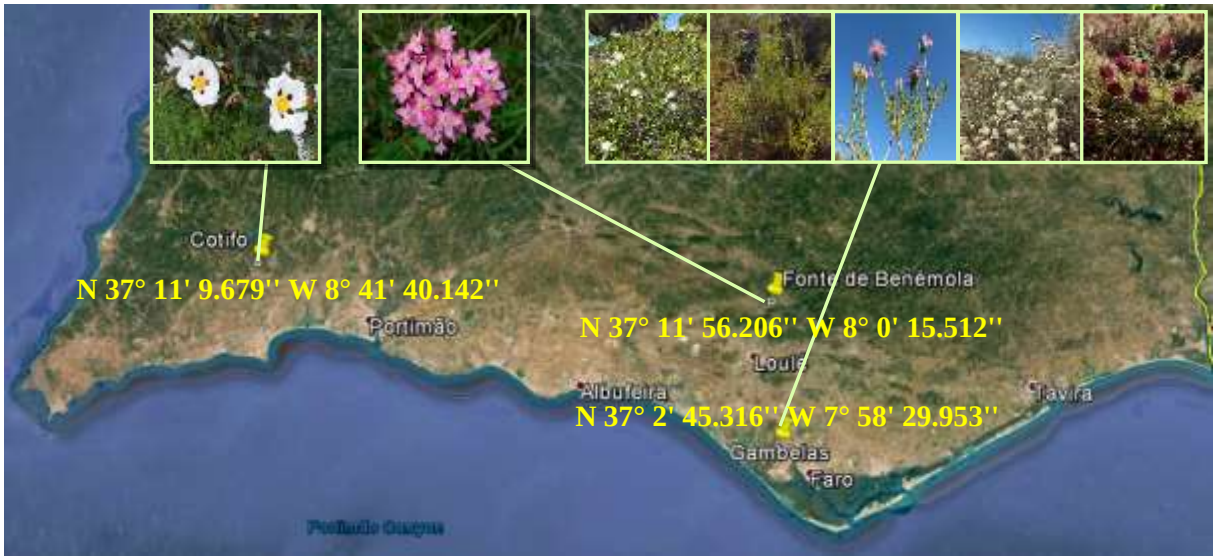


Figure 1 – Algarve map with the seven studied plants collection sites.
Cistus ladanifer in Cotifo – Lagos, *Centaurium erythraea* in Fonte de Benémola – Loulé, *Myrtus communis*, *Rosmarinus officinalis*, *Thymbra capitata; Thymus albicans* and *Thymus lotocephalus* in Gambelas – Faro.

These plants were selected to test the antioxidant and antidiabetic activity, since some of them were already mentioned in tradicional medicine and bibliography by having these activities (Table I).

Table I – Scientific and common names, code, voucher number and medicinal uses and properties of the seven plants studied.

Scientific name	Common name		Code	Voucher number	Medicinal uses and properties
	English	Portuguese			
Centaurium erythraea Rafn.	Small centaury	Fel-da-terra	CE	14270	Digestive, stomachic, tonic, depurative, sedative, antipyretic and antidiabetic [3,8,9,10];
Cistus ladanifer L.	Rockrose	Esteva	CL	14710	Aromatic, antibacterial, antifungal, skin diseases, antidiarrheic and anti-inflammatory[19]
Myrtus communis L.	Myrtle	Murta	MC	14304	Hypoglycemic, disinfectant, antiseptic, expectorant, antifungal, antibacterial and antioxidant[28,30];
Rosmarinus officinalis L.	Rosemary	Alecrim	RO	14272	Hepatoprotective, antibacterial, antithrombotic, antiulcerogenic, diuretic, antidiabetic, antioxidant and anti-inflammatory[36];
Thymbra capitata (L.) Cav.	Mediterranean thyme	Tomilho de Creta	TC	13492	Anti-inflammatory, antimicrobial, antioxidant, antiparasitical, respiratory infections, insecticidal and nematicidal[37];
Thymus albicans Hoffm. & Link	Thyme	Tomilho alvadio	TA	14303	Circulatory and digestive system, stomach and intestine problems, toothache, colds and regulate de menstrual cycle[37].
Thymus lotocephalus G. López & R. Morales	Thyme	Tomilho-cabeçudo	TL	14302	

1.1.1. *Centaurium erythraea* Rafn.

Figure 2 – *Centaurium erythraea* Rafn. plant.

Centaurium erythraea Rafn. is the autochthonous plant more recognize from the family Gentianaceae and it is widely distributed throughout the Mediterranean territory. It can be annual, biennial or perennial and blossoms from April to September. In Algarve it is possible to find it in clearing zones, dry, with poor and calcareous soils, where the competion is minor[(1)].

The stem can reach 50 cm tall and it is much branched, the leaves are pale green and smooth. The flower has a soft and characteristic aroma, five pink petals (which get white with the time) and can reach 8 mm length. The seeds are very small and formed within small capsules. The common portuguese name "fel-da-terra" it is due to its bitter taste that is referenced from classical antiquity. (http://www.spbotanica.pt/pmes/pmes4.html). It is refer in pharmacopoeias of 23 countries and was considered the "medicinal plant of the year" in 2004 by Springfield[(2-4)]. It is listed in the Council of Europe in category N2 as a natural source of food flavouring and can be added to food in small quantities[(3, 5)]. It is also used to prepare some commercial beverages due to its bitterness[(6)] and to preserve food due to its strong antimicrobial agents[(7)].

Medicinal applications and biological effects

This plant has been used for centuries to treat almost every kind of ailments. The infusions, decoctions and concoctions from the aerial parts are used in traditional medicine in many coutries. It is known for treat digestive problems, can be used as tonic to treat skin diseases, sedative, analgesic, depurative, among their antipyretic, anti-mutagenic, anti-inflammatory, anti-bacterial, anti-fungical and hypoglycaemic properties. It is also used to prevent, treat or control chronic diseases and muscle spasms in the intestinal tract[(3, 8, 9)]. It is also used to treat asthma, jaundice, intestinal parasitic infestation (anthelmintic), rheumatism,

urine retention, abdominal colic, fever and cardiac irregularity. There are many references to its antioxidant and radical scavenging activities[3], lower the triglyceride levels and total cholesterol[10], diuretic efficiency[11]. Some therapeutic effects are fungitoxic, choleretic, pancreatic and hepatoprotective and can prevent cardiovascular disorders[12]. All these properties are related to many phytochemical compounds present in *Centaurium erythraea* (CE) such as alkaloids, coumarins, triterpenes, phenolic acids and xanthones, xanthone derivatives, centauroside, centapicrin, flavonoids, gentiopicrin, gentiopicroside, isocoumarin, swertiamarin, triterpenes, wertiamarine, several steroids, essencial amino-acids and secoiridoids glucosides, the most important are gentiopicroside, swertiamarin and sweroside[2, 4, 6, 13]3. The main phenolic compounds present in centaury (CE) extract were several esters of hydroxycinnamic acids (*p*-coumaric, ferulic and sinapic acids)[14]. When administrated orally is not toxic on rats and protect against acetaminophen-induced hepatotoxicity[4], however certain studies claim that the doses recommended by healers are too high and can cause generalized congetion, degeneration and necrosis of both liver and kidneys[15].

1.1.2. *Cistus ladanifer L.*

Figure 3 – *Cistus ladanifer L.* plant.

Cistus ladanifer L. var. *maculatus* or rockrose is an autochthonous evergreen shrub, resinous and with a strong fragrance from family Cistaceae. Exists in all world but it is particularly abundant in all Mediterranean area and especially in the Portuguese territory. It has a resin, known as labdanum, rich in flavonoids and consists in a natural defense against herbivores because it causes mouth skeletal muscle relaxation through the inhibition of the calcium transport[16]. It is also used as a natural fixative in perfumery industry and recently associated to the sensorial quality of port wine[17, 18]. This shrub can reach 2.5 m height and width and prefers dry places with much sun, poor soils, open and degradated areas, especially for fires since its seeds are resistant. It is phytotoxic and inhibits the germination and growth of another plants[19]. The leaves are dark-green and between 3 and 10 cm length and 2 cm

width and are welded together at the base. The young leaves are a light green, bright and stickier. It blossoms from May to July, the flowers have 5 white petals with a purple spot and can reach 10 cm diameter. The fruit is a globular capsule with 7 to 10 compartments and that is the reason of its Greek name "ciste" which means box or basket. (http://www.mitra-nature.uevora.pt/Especies-e-habitats/Plantas/Lenhosas/Arbustos-e-Lianas/Cistaceae/Cistus-ladanifer-subsp.-ladanifer)

Medicinal applications and biological effects

Several studies were made with this plant since many years and it is known that is a rich source of flavonoids (quercetin, kaempferol and apigenin derivatives) and polyphenols wich show a large antioxidant and radical scavenging activities against peroxyl radicals in food and biological systems. Many phytochemical compounds were identified as vitamins and reducing sugars, useful against diseases caused by oxidative stress and for that reason it is appreciated in cosmetic industry. Polyunsaturated fatty acids, 72 terpenes (monoterpenes, sesquiterpenes and diterpenes), 43 phenylpropanoids and an additional 6 carbonylic compounds, alkaloids, polyacetylenes, tannins and steroids have been identified[19-21]. The labdanum exudate i tis secreted by glandular trichomes and it is rich in phytochemical compounds known for their antioxidante, anti-bacterial, anti-fungic, anti-cancer (citotoxic against pancreatic and breast cancer cells), anti-aggregant, anti-leukemic, cardio and dermo protective and myorelaxant properties[19].

In traditional medicine it is ingested through an infusion to treat digestive problems and colds, and the extracts used as sedative, hemostatic and anti-infective agent[22]. It treats several skin diseases, and it is antidiarrheics, anti-inflammatory, diarrhea, dysentery, menstruation discomfort, catarrh, antiseptic, astringent, tonic, expectorant, balsamic and emmenagogue. For cardiopathies, dyspneia, headache, insomnia, leukorrhea, myalgia, neuralgia, osteoarthritis, proctosis, rhinosis, sore, spasm, splenosis, ulcer and uterosis[19].

1.1.3. *Myrtus communis L.*

Figure 4 – *Myrtus communis* L. plant.

Myrtus communis L. known as myrtle belongs to myrtaceae family and is an aromatic evergreen shrub and grows mainly in Mediterranean climates. Is used since Greeks and Romans to treat diseases, as a flavouring and aphrodisiac[23]. Due to its bitter and intense flavour and aromatic properties, essential oils are used in food industry to enhance the flavour of meats, fish and souces and in bakery, in cosmetic industry is appreciated for increasing the tolerance and efficacy of retinol anti-anging products, in pharmaceutical industry and even to do a liqueur in Sardinia with the berries and leaves[24-26]. This shrub it is much branched and can reach 5 m tall. With dark green poited leaves that can reach 5 cm and 1.5 width. The flowers are white, with 5 petals and can reach 15 mm diameter. The fruits are pseudofruits, ellipsoids and blue dark or blacks with many seeds inside. The young leaves are edible like the fruits. It is resistant even in dry soils and high temperatures, it is appreciated as ornamental plant in gardens and public spaces.
(http://www.floraiberica.es/floraiberica/texto/pdfs/08_095_01%20Myrtus.pdf).

Medicinal applications and biological effects

The main phytochemical compounds present in myrtle (MC) are terpenes, terpenoids, phenyl-propanoids, phenolic compounds (such as myrtenol and myrtenol acetate[25]), hydrolysable tannins, anthocyanins and flavonoids. The fruits are a rich source of vitamins such as α-tocopherol[27-30].The leaves have the highest antioxidant activity and phenolic compounds in all plant[31]. These contents are known for the antioxidant and free radical scavenging activity, which promotes health and well-being. The essential oils are used to treat insomnia, and nervous conditions due to (-)-myrtenol which presents anxiolytic-like activity[32]. In tradicional medicine the leaves are used to treat wounds, haircare, diseases such as

inflammation and allergies[33], it is antiseptic, disinfectant and a regulator agent of hypoglycaemic. Also known due to its laxative and analgesic effect, treats orally infectious disease, skin diseases, breathing problems, psoriasis, gastrointestinal disorders, urinary infections, haemostatic, nephroprotective, and many more[28, 30, 34]. Ib bibliography many more characteristics such as anti-microbial, anti-fungic, anti-viral, anti-molluscicidal, insecticidal, protozoicidal, anti-atherogenicity, antidiabetic, antimutagenic, pro-apoptotic activity in cancer cells, cardiovascular activity, activity against recurrent aphthous stomatitis and hepatic ischemia, antiulcer[28, 30].

1.1.4. *Rosmarinus officinalis L.*

Figure 5 – *Rosmarinus officinalis* L. plant.

Rosmarinus officinalis L. belong to the Lamiaceae family and is a comum shrub in mediterranean known as rosemary. The leaves are edible and much appreciated in culinary due to its aromatic properties, especially in meats, barbecue, soup, sauces or in teas. It is a perennial plant which appreciates calcareous soils and it is widely used as ornamental. It blossoms from January to May and the flowers can be from purple until white. The name *rosmarinus* was given by the Romans, its Latin and it means “dew of the sea”. Its essential oils were used as unction, incense and in baths with religious purposes, and it is used as natural preservative of food, in cosmetic and phytocosmetic[35], aromatherapy, and medicinal treatments[36]. It is a persistent shrub that can grow until 1.8 m high, many branched, leaves are dark-green on the top and greyish green in the bottom and can reach the 2.5 cm long. They are small, opposite, and can go from linear to lanceolate. Each brunch can go from 5 to 15 flowers. (http://www.floraiberica.es/floraiberica/texto/pdfs/12_140_16_Rosmarinus.pdf)

Medicinal applications and biological effects

Studied since many years, this plant is a source of protein, fibers, vitamins and minerals[36]. Its essential oils are rich in phytochemical compouns such as monoterpenes and monoterpene hydrocarbons[37], phenolics diterpenes and acids, flavonoids and tannins. The antioxidant, anti-inflammatory and anticancer (prostate, breast, skin, leukemia and colon cancer[38]) capacity is mainly from the carnosic acid, carnosol e rosmarinic acid[39]. It showed acetylcholinesterase (AChE) inhibitory activity[40].

In tradicional medicine, rosemary was used to treat headache, inflamations, to treat wounds, to prevent bronchial asthma, as analgesic, anti-depressive, antirheumatic, stomach and breathing problems. Its biological effects are well study and the anti-bacterial, anti-fungic, antioxidant, anti-cancer, anti-inflammatory, anti-diabetic, antiulcerogenic, antinociceptive, antidepressant, antianxiety, antithrombotic, antiviral, antigiogenic and astringent are known[36]. The extracts have hypoglycaemic and antihyperglycaemic activity and inhibit the lipid peroxidation and activate the antiox enzymes. It is suggest in the potential treatment of obesity, high cholesterol and related diseases[41, 42]. It showed a bioinsecticidal effect and a potential therapie for Alzheimer´s disease[43, 44].

1.1.5. *Thymbra capitata* (L.) Cav., *Thymus albicans* Hoffm. & Link and *Thymus lotocephalus* G. López & R. Morales

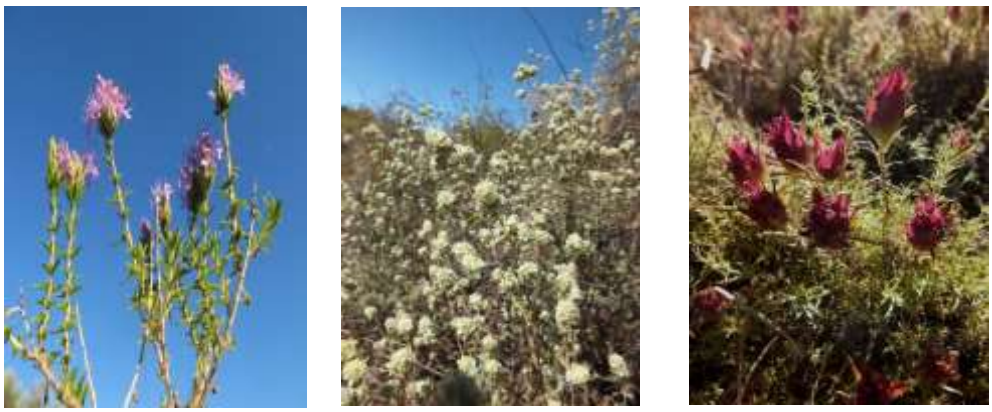


Figure 6 – *Thymbra capitata* (L.) Cav., *Thymus albicans* Hoffm. & Link and *Thymus lotocephalus* G. López & R. Morales plants.

Thyme species are small aromatic, evergreen and perennial herbs belonging to the genus *Thymus* L. from the family Lamiaceae. Many species are endemic of the Mediterranean area, including Portugal were is possible to find fourteen *taxa* including *Thymbra capitata*

(L.) Cav., *Thymus albicans* Hoffm. & Link and *Thymus lotocephalus* G. López & R. Morales 45. Thyme or thymus derives from the Greek "thyo" which means "sacrifice" (http://www.reherb.eu/en/content/thymbra-capitata).

These species are very versatile since they can be used green or dry in culinary as spices, can be ornamental and/or aromatizing or used for tradicional purposes. In Algarve they are used mainly in food as a spice in foods such as snails, saladas and grilled meats. Its essential oils are used to aromatized tooth paste, mouth wash, to preserve foods, in perfumery and in cosmetic industry.

Thymbra capitata or TC is a woody plant with many brunches that grows until 40 cm tall. It likes matos xerodílicos its brunches have small hairs, the leaves are from linear to lanceolate and can reach 10 mm long and 1.5 mm width. Groups of about 15 flowers blossom from May and June in the end of the brunches and goes until 10 mm and are purple. (http://www.floraiberica.es/floraiberica/texto/pdfs/12_140_20_Thymbra.pdf).

Thymus albicans or TA is a woody plant with brunches 45 cm high, leaves until 8 mm long and 3 mm width, eliptic, linear-spadulated, glabrous with little hairs. Its white flowers blossom in globes of 9 mm and the fruits are mericarpos ovoides e lisos. It grows in south west of Algarve in dry places, in glade pinewoods and near the coast in sandy soils. (http://www.floraiberica.es/floraiberica/texto/pdfs/12_140_21_Thymus.pdf)

Thymus lotocephalus or TL is an woody undershrub, endemic from the Algarve that grows until 30 cm. The floral brunches are larger, the leaves are linear and with small hairs, and could have until 10 mm long and 0.8 mm weidth. It blossoms fro April to May, in the coast or barrocal and the flowers are purple. (http://www.floraiberica.es/floraiberica/texto/pdfs/12_140_21_Thymus.pdf)

Medicinal applications and biological effects

In general all types of thymes produce secundary metabolites, recognized for the antioxidant capacities, anti-microbial, anti-tussic and to treat skin diseases[37]. In traditional medicine it is ingested as an infusion to treat gastro-intestinal problems and the essential oils have anti-fungical properties, can treat intestinal parasites and it has antipasmodic properties. TC is recognized for its high values of carvacrol, thymol e phenolics terpenes. Its infusions can treat respiratory infections, digestive problems, improves and purifies blood, to treat colds and regulate the menstrual cicle. The essential oils have antiacetylcholinesterase inhibitory

activity besides the antibacterial, antifungical, antioxidant, antispasmodic and anti-inflammatory properties48.

1.2. Antioxidants – reactive oxygen species and consequences

In human body many molecules and compounds interact. Free radicals are a consequence of the interaction of three molecules with our system: oxygen, nitrogen and sulfur, and the most known specie is derived from the element oxygen, which is essential for human life. The consequences of free radical are the reactive oxygens species (ROS). They have an unpaired electron higly instable and very susceptive to react with another molecules. These ROS include many forms of free radicals such as hydroxyl radical (·OH) and hydrogen peroxide (H2O2) amongs others.

The quality of life and habits such as smoking, drugs, pesticides and environmental pollutants are the main sources for the oxidative stress. Consequences of the oxidative stress caused by ROS are neurological disorders, adult respiratory distress syndrome, degenerative diseases associated with Alzheimer, diabetes mellitus, lupus erythematous among others.

Natural antioxidants are chemical compounds that even present in small quantities can inhibit theaction of free radical preventing oxidative stress and it consequences. Other than those natural antioxidants present in our body more can be aquire from the diet mainly consumption of plant products. Exemples of antioxidants include vitamins, phenolic compounds such as carotenoids, minerals, flavonoids, wich include flavonols, anthocyanins, among others. Antioxidants have the capacity to delay or inhibit the oxidative stress through the ability of interact with the free radicals and create new radicals more stable through intramolecular hydrogen bonding.

Due to the importance of antioxidants, several plants are studied every day to assess their healt benefit potentials.

1.3. Diabetes mellitus

Diabetes mellitus is a major endocrine disorder around the world and especially in western societies, were projections estimate around 380 million diabetics in the world for 2025 [38 RO]. Excess of ROS can cause apoptosis which damage β-cells and supress the insulin biosynthesis [8 RO]. In this disease the blood glucose levels are above normal and the body does not produce enough insulin hormone or it does not work as it should (helping sugar to go inside the cells).

(http://www.cdc.gov/diabetes/basics/diabetes.html).

Some drugs are available to treat DM such as acarbose. It inhibits the activities of enzymes related with the disease, for example α-amylase and α-glucosidase.

2. Objectives

- Evaluation of potential antioxidant and antidiabetic activity of seven aqueous extracts at four temperatures;
- Selection of the best temperature of extraction for the tested plant infusions;
- Selection of the best plant infusions for the tested temperatures;
- Check relations between all assays by correlation;
- Check for similarities between plant infusions, assays and temperatures using HCA.

3. Material and Methods

3.1. Chemicals

2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteau, Trolox, Gallic acid, Quercetin, sodium phosphate dibasic anhydrous, sodium dihydrogen phosphate dehydrate, ammonium molybdate, hydrochloric acid and α-amylase were purchased from Sigma-Aldrich Co. Ltd (United Kingdom); α-glucosidase was from Megazyme (Ireland); 2,4,6-tripyridyl-2-triazine (TPTZ), sodium carbonate, aluminium chloride, ethanol, potassium ferricyanide, sulfuric acid, glacial acetic acid and potassium persulfate were from Merck (Germany); 2-2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was from VWR (Portugal); tricloroacetic acid, ferric chloride, ascorbic acid and sodium hydroxide were from Prolab (Brazil); 3,5-Dinitrosalicylic acid (DNSA), 4-Nitrophenyl-α-D-glucopyranoside (pNGP) and acarbose were from Alfa Aesar (Germany); sodium chloride was from Laborspirit (Portugal); potassium sodium tartrate and potato starch were purchased from Panreac (Spain). All the chemicals and solvents used in this experiment were of analytical grade.

3.2. Equipment

The equipment used in this experiment was an Oven from Binder®, a Hotplate stirrer from VWR, an Explorer Pro digital balance from Ohaus®, a T70+ UV/VIS Spectrometer from PG Instruments, a Series 503 water bath from Nahita, a Vortex from Stuart®, a digital thermometer HI98501 Checktemp® from Hanna instruments and a micro pH 2001 from Crison.

3.3. Plant material

TC, TA, TL, RO and MC plants were collected in 2014 from the local area of University of Algarve, Campus of Gambelas - Faro (N 37° 2' 45.316" W 7° 58' 29.953"), CL was collected in 2010 in Cotifo - Lagos (N 37° 11' 9.679" W 8° 41' 40.142") and CE was collected in 2013 in Fonte de Benémola - Loulé (N 37° 11' 56.206" W 8° 0' 15.512").

The plants were identified and authenticated by Coronel José Rosa Pinto (UALG – Herbarium of University of Algarve) and a voucher specimen was created for these plants.

CL was dried in the oven at 45 ºC for two days. The rest of the plant materials were dried and stored at room temperature in a dry place protected from light.

3.4. Methodology

3.4.1. Extraction process

All the aerial parts of the dry material were reduced to coarse powder using a kitchen grinder and stored in a freezer at -20°C in capped plastic vials until use.

Infusions were made using 0.50 g of each plant in 20 mL of distilled water, previously heated and maintained at 25, 50, 75 and 95ºC for 15 min without stirring, filtered through Whatman® No. 4 paper and stored in capped eppendorfs at -20ºC until analysis. An aliquot of 2 mL in triplicate of each plant infusion were evaporated in the oven for the determination of dry weight.

3.4.2. Total phenolic content (TPC)

The total phenolic amount present in the plant infusions was determined using the Folin-Ciocalteau method (Huang et al., 2006). A calibration curve was prepared using Gallic acid as a standard and the results were expressed as milligrams of Gallic Acid Equivalents per gram of dry weight (mg GAE/g dw).

In this method, 0.10 mL of each properly diluted infusion were mixed with 0.50 mL of Folin-Ciocalteau´s reagent, 0.40 mL of saturated sodium carbonate solution (7.5% w/v) and then vortexed. After 30 min in the dark and at room temperature, the absorbance was read at 765 nm against a blank in a spectrometer. All the measurements were made in triplicate.

3.4.3. Total flavonoid content (TFC)

The method used was based on Lamaison and Carnat (1990). Quercetin was used as a standard through a calibration curve and the results were presented as milligrams of Quercetin Equivalents per gram of dry weight (mg QE/g dw).

To calculate the total flavonoid content, 0.40 mL of each infusion properly diluted were added to 0.80 mL of methanolic aluminium chloride solution (2% w/v) and then vortexed. The samples were left in the dark at room temperature for 10 min and the absorbance was read against a blank in a spectrometer at 430 nm. All the measurements were made in triplicate.

3.4.4. Total antioxidant activity (TAA)

The phosphomolybdenum method (Prieto et al., 1999) was used to determine the total antioxidant activity, a calibration curve with ascorbic acid was prepared as a standard and the results were expressed as milligrams of Ascorbic Acid Equivalents per gram of dry weight (mg AAE/g dw).

For that, 1 mL of a solution containing 0.60 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate were added to 0.10 mL of each plant infusion (after testing the correct dilution) and then vortexed. The samples were incubated in a thermal bath at 95ºC for 90 min, cooled to room temperature and the final absorbance measured (against a blank) at 695 nm in a spectrometer. All the measurements were made in triplicate.

3.4.5. Reducing power (RP)

The reducing power of each plant infusion was determined using the spectrophotometric method previously described by Oyaizu (1986). Trolox (standard) was used to prepare a calibration curve and the results were expressed as milligrams of Trolox Equivalents per gram of dry weight (mg TE/g dw).

To do this method, 0.25 mL of phosphate buffer (0.20 M, pH 6.6) previously prepared were mixed with 0.25 mL of potassium ferricyanide (1% w/v) and 0.10 mL of each properly diluted infusion. The solution was mixed, incubated at 50ºC for 20 min and then 0.25 mL of trichloroacetic acid (10% w/v) were added. After staying at room temperature for 10 min, 0.50 mL of the solution were mixed with 0.50 mL of distilled water and 0.10 mL of ferric chloride (0.1% w/v) and vortexed. With the aid of a spectrometer the absorbance was read at 700 nm against a blank. All the measurements were made in triplicate.

3.4.6. Ferric reducing antioxidant power (FRAP)

The ferric reducing antioxidant power was determined based on the method described by Benzie and Strain (1999). All the measurements were made in triplicate.

Briefly, fresh working solution was prepared by mixing 25 mL of 300 mM acetate buffer with 2.50 mL of 10 mM TPTZ solution and 2.50 mL of 20 mM ferric chloride solution. The working solution was heated to 37ºC before using. Then 0.10 mL of each diluted infusion were mixed with 0.90 mL of the FRAP working solution and vortexed. The samples were left in the dark at room temperature for 30 min and the absorbance was read at 593 nm (against a blank). The results were expressed as milligrams of Trolox Equivalents per gram of dry weight (mg TE/g dw).

3.4.7. DPPH free radical scavenging activity (DPPH)

The DPPH free radical scavenging activity was determined using the method described by Yen et al., (2000). The mean inhibitory concentration (concentration in dry weight of plant extract necessary to capture 50% of the free radicals in solution - IC_{50} in µg/mL) was determined by linear regression of % I and the infusion concentration, using the following equation $\%\, I = \frac{A_0 - A_t}{A_0} \times 100$ where A_0 is the absorbance of the control and A_t is the absorbance of the infusion. The concentrations of infusions were made to obtain at least two values of inhibition above and two below 50% in the linear part of the inhibition curve.

In this method, 0.50 mL of DPPH methanolic solution were added to 0.50 mL of each infusion (properly diluted) and vortexed. The samples were left in the dark at room temperature for 30 min and the absorbance was read at 517 nm in a spectrometer. All the measurements were made in triplicate.

3.4.8. ABTS free radical scavenging activity (ABTS)

According to Re et al., (1999), the ABTS free radical scavenging activity is determined through the IC_{50} (µg/mL), previously described for DPPH.

A stock solution of ABTS was prepared by spiking 25 mL of 7 mM ABTS with 0.44 mL of 140 mM potassium persulfate and was left in the dark at room temperature for 16 hours. Then this solution was diluted with ethanol just before use to an absorbance of 0.700 ± 0.02 nm at 734 nm. 1 mL of diluted ABTS solution was added to 0.05 mL of each properly diluted plant infusions and vortexed. The samples were left in the dark at room temperature for 5 min and the absorbance was read in a spectrometer at 734 nm. All the measurements were made in triplicate.

3.4.9. Colourimetric analysis

The method was described by Kelebek et al., (2008). All the infusions were diluted to the lowest value of concentration (around 0.003 g/mL) and a direct measurement of extract absorbance was made at 420, 520 and 620 nm against a blank in a spectrometer.

The following colour parameters were calculated:

Colour intensity	$CI = A_{420} + A_{520} + A_{620}$
Yellow proportion	$Ye\% = A_{420} * 100 / CI$
Red proportion	$Rd\% = A_{520} * 100 / CI$
Blue proportion	$Bl\% = A_{620} * 100 / CI$

3.4.10. α-amylase inhibition assay (α-amylase)

The α-amylase inhibition assay was based on the method described by Conforti et al., (2005) and modified by Sancheti et al., (2013). The IC_{50} (µg/mL) was determined as previously described for DPPH. In addition, a calibration curve was prepared using acarbose as a standard and the results were expressed as milligrams of Acarbose Equivalents per gram of dry weight (mg AcE/g dw).

To start, a 0.02 M phosphate buffer (pH 6.9 containing 6.70 mM NaCl) and a colour reagent solution (sodium potassium tartrate in 2 M NaOH with 96 mM 3,5-dinitrosalicylic acid) were previously prepared. A starch solution (2% w/v) was made with the phosphate buffer and 0.10 mL were mixed with 0.05 mL of each infusion and incubated for 10 min at room temperature. To this mixture, 0.10 mL of enzyme solution (2.5 U/mL) made in phosphate buffer were added and incubated for 5 min at room temperature, followed by the addition of 0.10 mL of colouring reagent and vortexed. The mixture stayed for 15 min in a water bath at 95ºC, was cooled to room temperature and 0.90 mL of distilled water were added. The absorbance of the samples was read at 540 nm in a spectrometer. All the measurements were made in triplicate.

3.4.11. α-glucosidase inhibition assay (α-glucosidase)

Like in α-amylase, a calibration curve with acarbose was used as a standard and the method was based on Li HL et al., (2009) and modified by Deepak Kumar et al., (2011). The results were expressed as milligrams of Acarbose Equivalents per gram of dry weight (mg AcE/g dw).

Three solutions were previously prepared, a 0.10 M phosphate buffer (pH 6.8), a 0.20 M sodium carbonate solution and a 0.50 M pNGP solution. Then, 0.15 mL of each properly diluted infusion was mix with the same volume of enzyme solution containing 0.50 U/mL and incubated at 37 ± 1ºC for 10 min. After the incubation 0.15 mL of the substrate pNGP (0.50 mM concentration in 0.10 M phosphate buffer, pH 6.8) was added to the mixture, allowed to incubate at 37 ± 1ºC for 30 min. The reaction was terminated by the addition of 0.60 mL of 0.20 M sodium carbonate solution and the absorbance of the solution produced was recorded at 405 nm in a spectrometer. All the measurements were made in triplicate.

3.4.12. Statistical analysis

Excel (Microsoft Office 2013) from Microsoft© was used to treat the data.

All the experiments were made in three replicates and the results expressed as the mean ± standard deviation.

SPSS® 22 from IBM® was used to analyse all data through the analysis of variance procedure to determine significant differences between the results. ANOVA with the post-hoc test LSD (Least Significant Difference) or Games-Howell was used, after checking the homogeneity of variances with Levene's test. To determine relationships between the assays, Pearson's correlations were determined. Finally, a Hierarchical Cluster Analysis (HCA) was applied to results in order to combine the different plants and temperatures by similarity in a dendrogram. The cluster forming method is through group linkage and measures the squared Euclidean distance. Relations between the assays were also investigated after the results were standardized using z-score.

4. Results and Discussion

4.1. Phenolic compounds and antioxidant activity

4.1.1. Total phenolic and flavonoid content

The TPC was the first assay done and it allows us to determine the total phenolic content of the infusions using the Folin-Ciocalteau. According to the results shown in Table II, the plant infusions with the highest TPC values on average were MC and CL with values averaging around 375 and 320 mg GAE/g dw. These plants were followed by TA > TL > RO > TC (the four aromatic plants) and finally CE was the plant with the lowest average value, approximately 45 mg GAE/g dw. For this assay MC was also the best at the four temperatures tested. Infusions obtained at 75ºC provided the highest TPC values for each plant, except for TC at 95ºC. The lowest values were obtained at 25 and 50ºC. Through the statistical analysis of the results, at each temperature and between the seven plants, it was possible to observe that only CE and TC infusions were statistically different ($p<0.05$) to every other plant. TL was the only one always similar ($p>0.05$) to some other plant infusion at all temperatures. TA and TL had similarities at 25ºC, the same was true for CL with MC and TL with both RO and TA at 50ºC. Finally, similarities were also found between RO and TL at the temperatures of 75 and 95ºC.

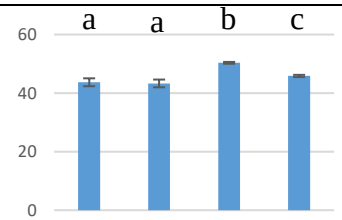
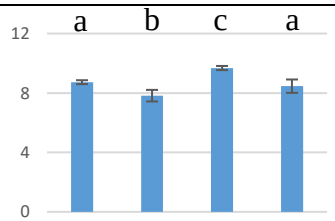
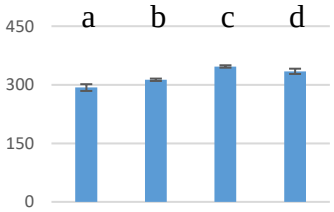
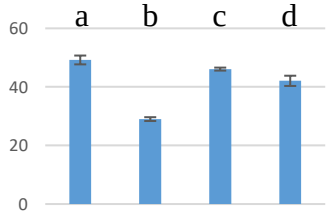
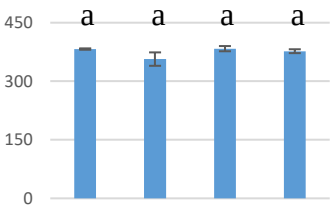
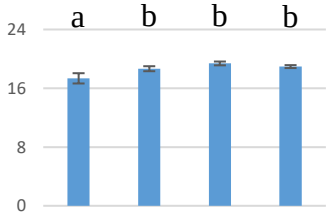
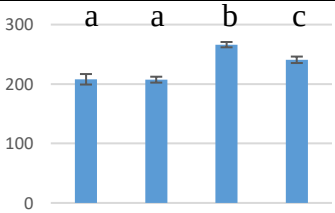
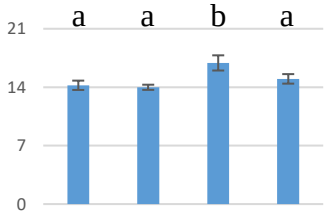
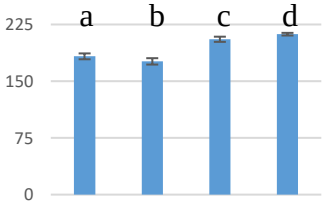
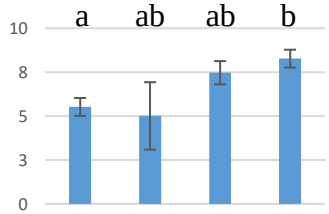
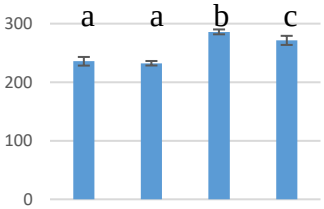
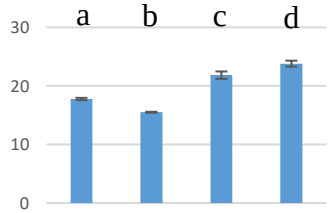
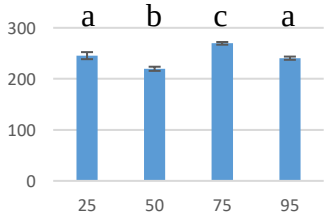
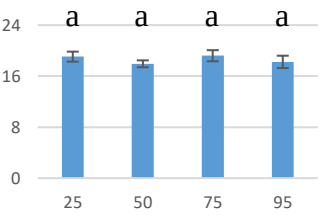
The behaviour of the plants regarding the effect of the temperature on the infusions obtained was studied analysing the statistical variation of the means obtained for each temperature in each plant. On Table III is possible verify that CE, RO and TA infusions had the exact same behaviour, no difference between the TPC values of infusions obtained at 25 and 50ºC, then at 75ºC there was a value increase, followed by a decrease at 95ºC. The only difference in CL was that 50ºC was different from the other temperatures and higher than 25ºC and TL showed that the temperatures of 25 and 95ºC were statistically similar. TC had the same behaviour than the previous ones except an increase in the values from 75 to 95ºC. Finally, for MC there was no difference between the results obtained at any temperature. Statistically, both CL and TC infusions were different ($p<0.05$) between themselves at all temperatures, which indicates that temperature influences the extraction of phytochemical compounds present in these two plants (in this case that react with the Folin reagent), while for MC all temperatures were similar ($p>0.05$), which hints temperature had no effect in the extraction. In that case this statistical analysis is important to understand how and if the extraction temperature influences the results. For instance, in Table III there was a decrease from 25 to 50ºC in MC values but without statistical significance.

Table II – TPC and TFC of the seven plant infusions obtained at four different temperatures.

	T (°C)	Plant						
		CE	CL	MC	RO	TC	TA	TL
TPC (mg GAE/g dw)	25	43.70 ± 1.35^{a}	292.77 ± 8.65^{b}	382.40 ± 1.74^{c}	207.95 ± 8.82^{d}	182.87 ± 3.88^{e}	235.72 ± 7.38^{f}	245.48 ± 6.91^{f}
	50	43.31 ± 1.32^{a}	312.73 ± 3.06^{b}	356.72 ± 17.17^{b}	207.34 ± 4.95^{c}	176.22 ± 4.21^{d}	232.33 ± 3.85^{e}	219.69 ± 4.10^{ce}
	75	50.34 ± 0.31^{a}	346.77± 3.06^{b}	383.47 ± 6.66^{c}	266.21 ± 4.48^{d}	205.45 ± 3.43^{e}	285.91 ± 4.17^{f}	269.63 ± 2.65^{d}
	95	45.85 ± 0.38^{a}	334.37 ± 6.76^{b}	376.96 ± 4.95^{c}	240.73 ± 5.43^{d}	212.18 ± 1.76^{e}	271.40 ± 7.77^{f}	240.50 ± 3.13^{d}
Average		**45.80 ± 3.23**	**321.66 ± 23.85**	**374.89 ± 12.44**	**230.56 ± 28.43**	**194.18 ± 17.33**	**256.34 ± 26.48**	**243.83 ± 20.51**
TFC (mg QE/g dw)	25	8.73 ± 0.13^{a}	49.21 ± 1.51^{b}	17.35 ± 0.70^{c}	14.23 ± 0.56^{d}	5.52 ± 0.51^{e}	17.77 ± 0.20^{cf}	19.05 ± 0.79^{f}
	50	7.82 ± 0.39^{a}	29.01 ± 0.65^{b}	18.66 ± 0.34^{c}	13.99 ± 0.31^{d}	5.01 ± 1.92^{ad}	15.51 ± 0.08^{e}	17.93 ± 0.54^{ce}
	75	9.68 ± 0.14^{a}	46.09 ± 0.51^{b}	19.38 ± 0.26^{c}	16.91 ± 0.91^{d}	7.47 ± 0.66^{e}	21.85 ± 0.63^{f}	19.20 ± 0.87^{c}
	95	8.46 ± 0.45^{a}	42.08 ± 1.75^{b}	18.95 ± 0.20^{c}	15.00 ± 0.57^{d}	8.27 ± 0.51^{a}	23.81 ± 0.51^{e}	18.23 ± 0.97^{cd}
Average		**8.67 ± 0.77**	**41.60 ± 8.88**	**18.59 ± 0.87**	**15.03 ± 1.32**	**6.57 ± 1.55**	**19.74 ± 3.78**	**18.60 ± 0.62**

The results are presented as mean ± standard deviation (of the triplicates). Average is presented as mean ± standard deviation of the results obtained at the four temperatures (mean of each temperature). Different letters in the same row correspond to statistically different results ($p<0.05$). TPC, total phenolic content; TFC, total flavonoid content; GAE, gallic acid equivalents; QE, quercetin equivalents; dw, dry weight. CE, *C. erythraea;* CL, *C. ladanifer*; MC, *M. communis*; RO, *R. officinalis*; TC, *T. capitata*; TA, *T. albicans*; TL, *T. lotocephalus.*

Table III - Graphical representation of temperature influence in TPC (mg GAE/g dw) and TFC (mg QE/g dw) results. Values obtained in each assay (y axis) at 25, 50, 75 and 95°C (x axis). Different letters in each graph correspond to statistically different results ($p<0.05$).

Plant	TPC	TFC
C. erythraea CE		
C. ladanifer CL		
M. communis MC		
R. officinalis RO		
T. capitata TC		
T. albicans TA		
T. lotocephalus TL		

In bibliography it is mentioned that phenolics are frequently related with the antioxidant capacity, since CL and MC presented a higher total phenolic content it is expected that they will also have a strong antioxidant activity. The temperature of 75ºC was the best to extract these phenolic compounds from the dry plants and 25 and 50ºC the worst temperatures.

Flavonoids (polyphenols) are a specific subclass that belongs to the group of phenolic compounds and are known mainly for their properties against oxidative stress. In the next assay, TFC was determined and better results were obtained from CL infusion that presented the highest value at all temperatures, with values averaging around 40 mg QE/g dw, followed by TA > TL > MC > RO > CE and exceptionally TC which had the lowest value, around 6 mg QE/g dw (Table II). Once again, plant infusions presented in general the highest TFC values at 75ºC. Exceptions were CL at 25ºC and TA at 95ºC which were the best extraction temperatures, and once again at both 25 and 50ºC the worst. In this assay, only the CL infusion had statistical differences ($p<0.05$) with all plants and at all temperatures. Once again TL and, in this case, MC were statistically similar ($p>0.05$) to some other plant at all temperatures. Regarding the extraction temperatures, similarities at 25ºC were found between both MC and TA and this last one to TL. At 50ºC similarities existed between TC with both CE and RO, and TL with MC and TA. MC infusion was similar to TL at 75ºC and finally at 95ºC, similarities were found between CE and TC, and TL with both MC and RO.

Regarding the behaviour (Table III), CE and CL values decreased from 25 to 50ºC and from 75 to 95ºC with an increase from 50 to 75ºC, although CE at 25 and 95ºC was statistically similar. In RO infusion there was no difference between 25, 50 and 95ºC, while TA had an increase from 75 to 95ºC. For TL infusion the variation of the assay values was not significant and for TC the only significant variation was between 25 and 95ºC. For MC the only difference was an increment from 25 to 50ºC. Analysing the results obtained at all temperatures, CL and TA infusions were different ($p<0.05$) between themselves while TL had no differences.

The ratio between total phenols and total flavonoids (TPC:TFC) was calculated (data not shown) and was highest in CE infusion and lowest in TC infusion. Those results show that the amount of TFC was higher in CE infusion when compared with the other six plant infusions.

On TPC assay the infusions with the extraction temperature of 25ºC were the most statistically similar with the other infusion temperatures between all plants, while in this assay was the least statistically similar.

4.1.2. Total antioxidant activity, reducing power and ferric reducing antioxidant power

CL and MC were the plant infusions with the highest TAA results (average around 500 mg AAE/g dw) followed by TL > TA > RO > TC and once again CE was the lowest (average approximately 235 mg AAE/g dw) (Table IV). At 25 and 95ºC MC infusion showed the highest total antioxidant activity, while at the extraction temperatures of 50 and 75ºC CL was the one with the highest value. In this assay, each extraction temperature was the best for at least one plant infusion but generally, 75ºC and both 25 and 50ºC were once again the best and the worst temperatures respectively. Statistically CL, RO and TL were always similar ($p>0.05$) to some other plant at all temperatures. Analysing similarities at each temperature, these were found between both RO with TC, and CL with TL at 25ºC. At 50, 75 and 95ºC similarities were found between both CL with MC and between RO, TA and TL, additionally CE with TC were only similar at 50 and 95ºC.

Regarding the plant infusions behaviour (Table V), TA and TC values had a decrease from 25 to 50ºC followed by an increase at 75ºC and lastly a decrease at 95ºC for TA, while TC presented no significant difference between 75 and 95ºC. Both CL and MC increased from 25 to 50ºC and decreased in the rest of the temperatures however for CL no difference was found between 50 and 75ºC. CE had an increase of values from 25 to 95ºC although statistically both 50 and 75ºC and this last one with 95ºC are not different. In TL a decrease was noticed from 25 to 50ºC, followed by an increase at 75 and again a decrease at 95ºC but statistically only at 75 and 95ºC the values were different. Finally RO had only one statistical difference, between 50 and 75ºC. It should be noted that TA was the only plant that showed statistical differences ($p<0.05$) between all temperatures.

Through the analysis of RP results in Table IV, it was possible to observe that MC infusion had the highest average at all temperatures with values around 510 mg TE/g dw, followed by CL > TL > RO > TA > TC and once again CE with approximately 30 mg TE/g dw was the lowest. Once again infusions presented higher results at 75ºC and at 25 and 50ºC the lowest values, except for MC at 95ºC (lowest). Statistically, both CE and MC were different ($p<0.05$) from all plants at all temperatures. At 25ºC RO was similar ($p>0.05$) to TC and TA and at 50ºC CL was similar with the aromatic plants (*Rosmarinus* - RO, both *Thymus* – TA and TL and *Thymbra* - TC). At 75ºC similarities were found between RO and TA with both TC and TL, CL with TL and at 95ºC TA with TL

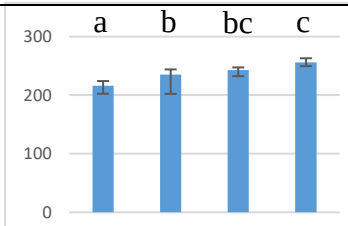
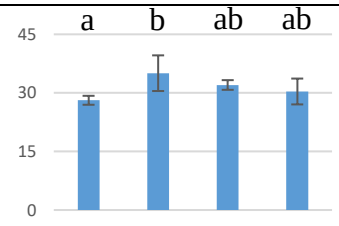
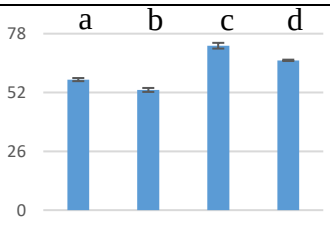
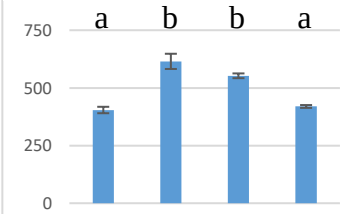
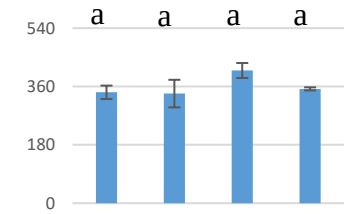
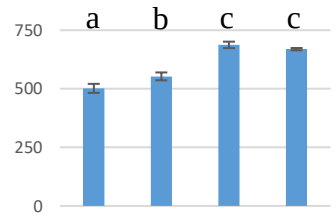
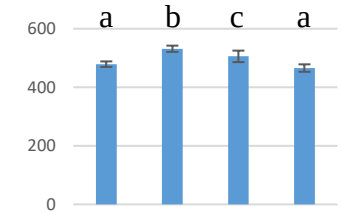
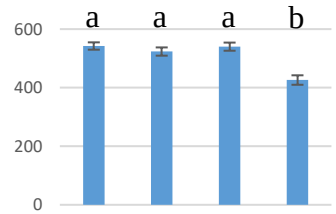
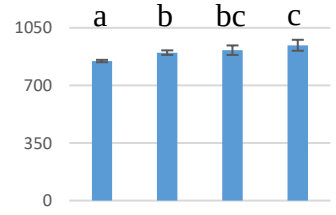
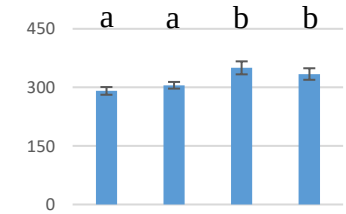
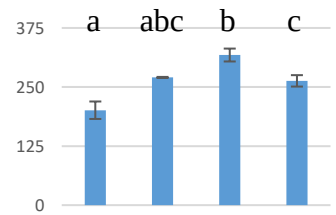
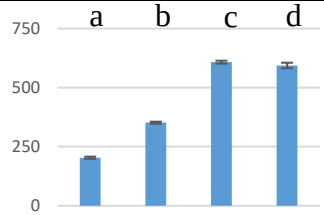
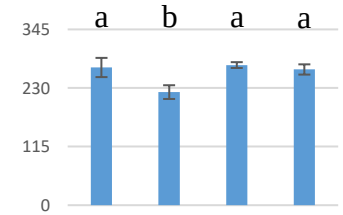
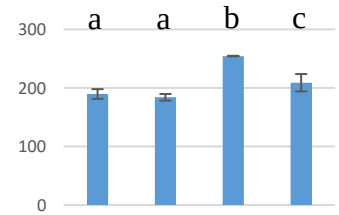
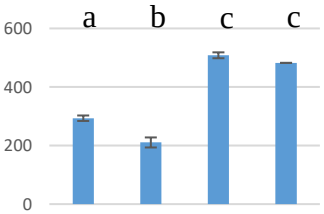
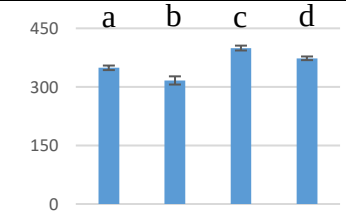
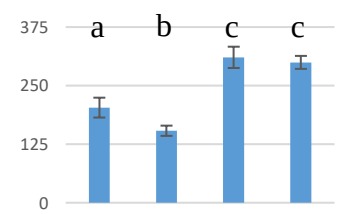
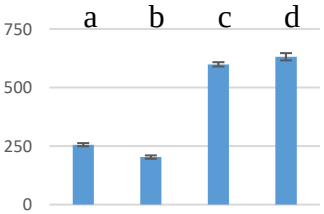
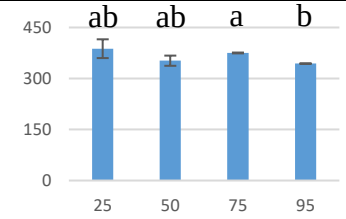
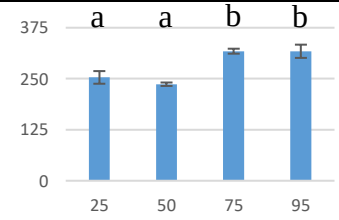
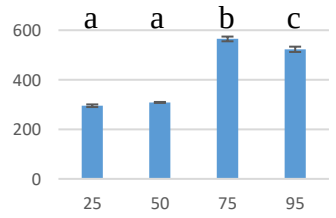
.

Table IV – TAA, RP and FRAP of the seven plant infusions obtained at four different temperatures.

	T (ºC)	Plant						
		CE	CL	MC	RO	TC	TA	TL
TAA (mg AAE/g dw)	25	216.33 ± 7.81^{a}	404.18 ± 13.91^{b}	478.98 ± 9.49^{c}	290.97 ± 9.85^{d}	270.25 ± 18.85^{d}	348.90 ± 5.82^{e}	387.68 ± 27.62^{b}
	50	235.06 ± 8.99^{a}	615.05 ± 32.95^{b}	531.63 ± 10.66^{b}	305.44 ± 8.53^{c}	221.96 ± 13.48^{a}	316.52 ± 10.50^{c}	352.37 ± 14.95^{c}
	75	242.77 ± 4.61^{a}	552.48 ± 10.30^{b}	505.64 ± 19.64^{b}	350.02 ± 16.62^{c}	275.01 ± 5.71^{d}	399.52 ± 6.21^{c}	375.12 ± 1.27^{c}
	95	255.74 ± 7.29^{a}	419.55 ± 6.09^{b}	465.69 ± 12.74^{b}	334.20 ± 14.74^{cd}	266.38 ± 10.05^{a}	373.23 ± 4.68^{c}	343.83 ± 0.76^{d}
Average		**237.48 ± 16.48**	**497.82 ± 102.67**	**495.49 ± 29.27**	**320.16 ± 26.82**	**258.40 ± 24.55**	**359.54 ± 35.35**	**364.75 ± 20.20**
RP (mg TE/g dw)	25	28.11 ± 1.15^{a}	342.11 ± 20.68^{b}	542.32 ± 12.53^{c}	200.94 ± 18.48^{d}	189.66 ± 8.30^{d}	203.06 ± 21.21^{d}	253.16 ± 15.48^{e}
	50	35.05 ± 4.57^{a}	338.19 ± 42.56^{bde}	523.44 ± 14.06^{c}	270.26 ± 1.16^{b}	183.99 ± 5.63^{d}	153.67 ± 10.94^{d}	236.47 ± 4.19^{e}
	75	32.03 ± 1.24^{a}	409.40 ± 23.11^{b}	540.15 ± 13.92^{c}	317.76 ± 13.64^{de}	254.38 ± 0.48^{d}	310.38 ± 22.73^{de}	317.43 ± 6.10^{be}
	95	30.36 ± 3.31^{a}	352.69 ± 4.77^{b}	426.14 ± 16.18^{c}	263.08 ± 12.09^{d}	208.92 ± 14.82^{e}	299.62 ± 13.84^{f}	317.25 ± 16.21^{f}
Average		**31.39 ± 2.92**	**360.60 ± 33.11**	**508.01 ± 55.23**	**263.01 ± 47.97**	**209.24 ± 31.93**	**241.68 ± 75.97**	**281.08 ± 42.42**
FRAP (mg TE/g dw)	25	57.70 ± 0.69^{a}	501.87 ± 19.28^{b}	848.13 ± 7.21^{c}	203.28 ± 4.69^{d}	293.40 ± 9.35^{e}	255.94 ± 6.67^{f}	295.19 ± 5.34^{e}
	50	53.13 ± 0.88^{a}	552.98 ± 16.87^{b}	899.25 ± 13.77^{c}	351.66 ± 4.17^{d}	210.62 ± 17.16^{e}	202.95 ± 7.13^{e}	308.48 ± 1.99^{f}
	75	72.68 ± 1.26^{a}	687.51 ± 13.85^{b}	914.11 ± 28.80^{c}	608.06 ± 6.13^{d}	508.33 ± 10.03^{e}	598.81 ± 9.24^{df}	564.82 ± 9.58^{f}
	95	66.22 ± 0.30^{a}	669.30 ± 4.90^{b}	943.93 ± 33.37^{c}	593.75 ± 11.61^{d}	482.32 ± 0.22^{e}	631.25 ± 15.45^{bd}	522.95 ± 10.73^{e}
Average		**62.43 ± 8.72**	**602.92 ± 89.94**	**901.36 ± 40.05**	**439.19 ± 196.40**	**373.67 ± 144.88**	**422.24 ± 224.06**	**422.86 ± 140.89**

The results are presented as mean ± standard deviation (of the triplicates). Average is presented as mean ± standard deviation of the results obtained at the four temperatures (mean of each temperature). Different letters in the same row correspond to statistically different results between different plants at the same temperature and assay ($p < 0.05$). TAA, total antioxidant activity; RP, reducing power; FRAP, ferric reducing antioxidant power; AAE, ascorbic acid equivalents; TE, trolox equivalents; dw, dry weight. CE, *C. erythraea;* CL, *C. ladanifer*; MC, *M. communis*; RO, *R. officinalis*; TC, *T. capitata*; TA, *T. albicans*; TL, *T. lotocephalus.*

Table V - Graphical representation of the means with standard deviations obtained from three replicates in TAA (mg AAE/g dw), RP (mg TE/g dw) and FRAP (mg TE/g dw) assays (y axis) at 25, 50, 75 and 95°C (x axis).

Plant	TAA	RP	FRAP
C. erythraea CE			
C. ladanifer CL			
M. communis MC			
R. officinalis RO			
T. capitata TC			
T. albicans TA			
T. lotocephalus TL			

On Table V it is possible to observe that TL and TA had almost the same behaviour, a decrease in values from 25 to 50ºC (not significant for TL) and an increase at 75ºC which had equal values to 95ºC. For TC 25 and 50ºC were statistically similar, an increase was observed at 75ºC finishing in a decrease at 95ºC.

For both MC and CL there was no variation in the results except a decrease for MC from 75 to 95ºC. CE infusion values increased from 25 to 50ºC and maintained throughout the remaining temperatures while RO increased until 75 and finished with a decrease at 95ºC even though all temperatures are statistically similar. Contrasting with the other plants and previous assays, temperature seemed to have no statistically significant effect ($p>0.05$) on CL infusion.

In FRAP assay, MC was once again the plant with the highest values at all temperatures, averaging around 900 mg TE/g dw, followed by CL > RO > TL > TA > TC and finally CE with approximately 60 mg TE/g dw showed the inferior value (Table IV). Once again 75ºC and both 25 and 50ºC showed to be the best and worst temperatures of extraction respectively, except for the infusion from TA at 95ºC (best). CE and MC infusions were the only ones statistically different ($p<0.05$) from all plants at all temperatures. TC was statistically similar ($p>0.05$) to TL at 25ºC and at 50ºC to TA. At 75ºC TA was similar to RO and TL and lastly at 95ºC RO was similar with TA, and this with CL, as well TC with TL.

Regarding the plant infusions behaviour (Table V), TA, TC, CE and TL had a decrease from 25 to 50ºC (for TL there was no variation), followed by an increase at 75ºC and again a decrease at 95ºC (for TC there was no variation while TA increased). For CL and RO both had similar behaviour which was an increase until 75ºC and then CL had no variation from this to 95ºC while RO had a decrease. MC infusion presented an increase from 25 to 95ºC although both 50 and 75ºC and this with 95ºC are not statistically different. The effect of temperature was mostly noted on CE, RO and TA which were statistically different ($p<0.05$) at all temperatures.

The aromatic plants (*Rosmarinus* - RO, both *Thymus* – TA and TL and *Thymbra* - TC) had more statistical similarities between themselves, in these three assays, than with the other plants. The assay most influenced by the temperature was FRAP, where three of the seven plants were statistically different at all temperatures.

4.1.3. DPPH and ABTS free radical scavenging activity

The radical scavenging activity was determined using the DPPH and ABTS radicals.

Since the results were presented as IC_{50}, which corresponds to the concentration of plant extract necessary to capture 50% of the free radicals in solution, lower values mean a higher activity.

Analysing both DPPH and ABTS results on Table VI, once again MC and CL were the best plants with the lowest IC_{50} values at all temperatures, with an average of 14 and 68 µg/mL for MC and 19 and 102 µg/mL for CL, respectively. In the middle values there were the four aromatic plants (*Rosmarinus* - RO, both *Thymus* – TA and TL and *Thymbra* - TC) and CE was the worst plant with the highest IC_{50} value, approximately 370 and 1380 µg/mL, respectively. Once again MC infusion values were the highest at all temperatures in both assays. Above 25ºC there was a higher extraction power of phytochemicals with DPPH radical scavenging activity, particularly 75 and 95ºC. The highest values of antiradical activities against ABTS were obtained for extraction temperatures of 75ºC or above except for CE (50ºC). Although the two free radicals used were different and showed different affinities towards the plants, for the temperatures of 75 and 95ºC these two assays where more times similar between them than the other assays and kept the relation between the plants (CE different from the rest of the plants and with the worst values, the aromatic plants similar between them and MC and CL with the best values). CE infusion was always statistically different ($p<0.05$) from the other plants, MC was always statistically similar ($p>0.05$) to CL and TA to TL and the same was verified between all the aromatic plants (*Rosmarinus* - RO, both *Thymus* – TA and TL and *Thymbra* - TC) at 75 and 95ºC, which show us that higher temperatures disguise the differences between plants.

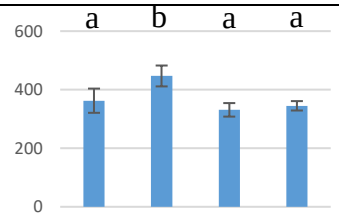
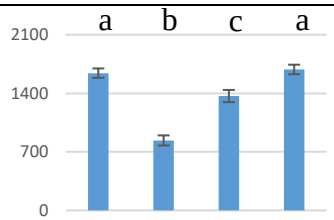
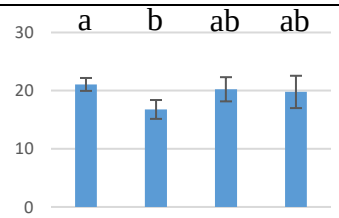
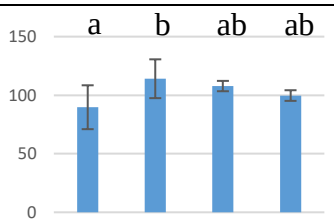
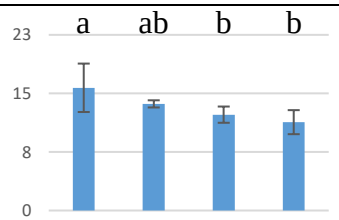
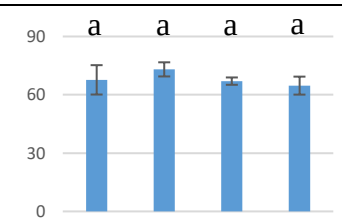
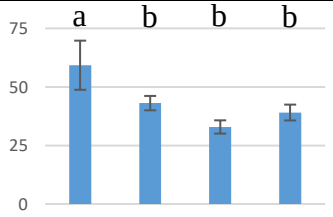
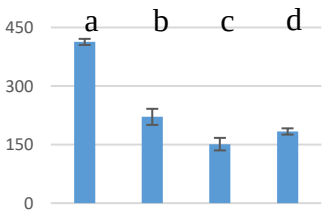
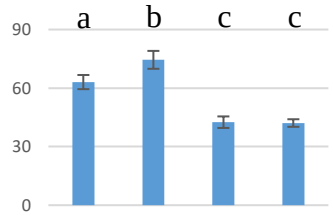
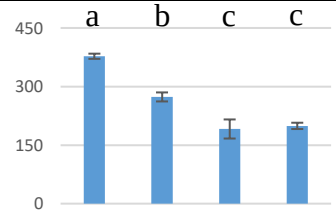
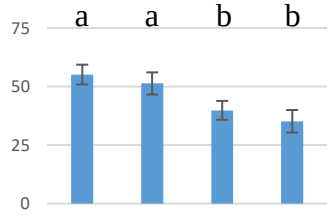
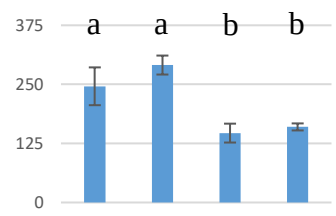
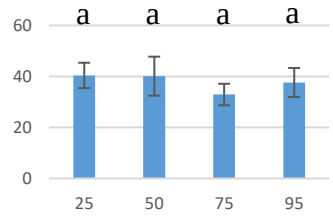
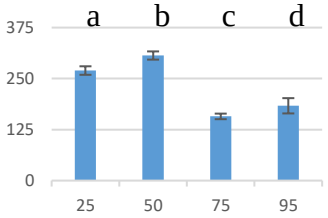
Regarding the behaviour in both assays (Table VII) it was possible to observe that in CE there was one statistical difference at 50ºC ($p<0.05$), which corresponded to the highest value in DPPH IC_{50} and the opposite in ABTS IC_{50}, where 50ºC followed by 75ºC corresponded to the lowest values. In CL the behaviour was the same for both assays, 25 and 50ºC were different between them although similar ($p>0.05$) with the rest of the temperatures. Once again the value at 50ºC corresponded to the lowest and the highest IC_{50} for DDPH and ABTS respectively. Concerning MC in DPPH, the values decreased from 25 to 75ºC but statistically 50ºC was similar to all temperatures. In ABTS all temperatures gave similar values. In RO the values decreased from 25 to 75ºC in both assays and increased at 95ºC.

Table VI – DPPH and ABTS radical scavenging activities IC_{50} of the seven plant infusions obtained at four different temperatures.

	T (°C)	Plant						
		CE	CL	MC	RO	TC	TA	TL
DPPH IC_{50} (µg/mL)	25	362.37 ± 41.37^{a}	21.07 ± 1.11^{bd}	15.72 ± 3.10^{b}	59.27 ± 10.49^{bcd}	63.08 ± 3.63^{c}	55.09 ± 4.23^{cd}	40.40 ± 4.97^{d}
	50	446.90 ± 35.65^{a}	16.77 ± 1.62^{b}	13.66 ± 0.46^{b}	43.07 ± 3.07^{c}	74.46 ± 4.59^{d}	51.31 ± 4.72^{c}	40.12 ± 7.64^{bc}
	75	331.19 ± 23.07^{a}	20.24 ± 2.08^{bc}	12.28 ± 1.04^{b}	32.90 ± 2.83^{cd}	42.53 ± 2.95^{d}	39.81 ± 4.03^{d}	32.90 ± 4.22^{cd}
	95	345.00 ± 16.04^{a}	19.78 ± 2.78^{b}	11.32 ± 1.54^{b}	39.06 ± 3.38^{c}	42.09 ± 1.94^{c}	35.14 ± 4.82^{c}	37.62 ± 5.70^{c}
Average		**371.37 ± 51.95**	**19.47 ± 1.87**	**13.25 ± 1.91**	**43.58 ± 11.27**	**55.54 ± 15.97**	**45.34 ± 9.40**	**37.76 ± 3.47**
ABTS IC_{50} (µg/mL)	25	1640.88 ± 56.43^{a}	89.73 ± 18.74^{b}	67.69 ± 7.56^{b}	413.06 ± 7.82^{c}	377.56 ± 6.64^{c}	245.91 ± 40.00^{d}	269.67 ± 10.43^{d}
	50	836.48 ± 60.32^{a}	114.06 ± 16.57^{b}	73.08 ± 3.64^{b}	221.09 ± 20.56^{c}	273.37 ± 11.56^{d}	290.82 ± 20.05^{d}	306.53 ± 10.06^{d}
	75	1366.99 ± 73.02^{a}	107.80 ± 4.41^{bc}	67.03 ± 1.89^{b}	151.18 ± 16.05^{cd}	191.28 ± 24.45^{d}	146.83 ± 20.01^{cd}	157.28 ± 6.83^{cd}
	95	1684.94 ± 57.20^{a}	99.70 ± 4.55^{b}	64.72 ± 4.63^{b}	183.63 ± 7.99^{c}	199.23 ± 8.04^{c}	159.90 ± 7.44^{c}	183.33 ± 18.72^{c}
Average		**1382.32 ± 390.13**	**102.82 ± 10.52**	**68.13 ± 3.54**	**242.24 ± 117.41**	**260.36 ± 86.44**	**210.87 ± 69.09**	**229.20 ± 70.46**

The results are presented as mean ± standard deviation (of the triplicates). Average is presented as mean ± standard deviation of the results obtained at the four temperatures (mean of each temperature). Different letters in the same row (right side) correspond to statistically different results between different plants at the same temperature and assay ($p < 0.05$). DPPH, DPPH free radical scavenging activity; ABTS, ABTS free radical scavenging activity. CE, *C. erythraea;* CL, *C. ladanifer*; MC, *M. communis*; RO, *R. officinalis*; TC, *T. capitata*; TA, *T. albicans*; TL, *T. lotocephalus.*

Table VII - Graphical representation of the means with standard deviations of the IC_{50} values obtained from three replicates in DPPH (µg/mL) and ABTS (µg/mL) assays (y axis) at 25, 50, 75 and 95°C (x axis).

Plant	DPPH	ABTS
C. erythraea CE		
C. ladanifer CL		
M. communis MC		
R. officinalis RO		
T. capitata TC		
T. albicans TA		
T. lotocephalus TL		

Statistically the temperature of 25ºC was different from the others in DPPH while in ABTS all temperatures were different. On TC there was an increase from 25 to 50ºC and a decrease to 75 which was statistically similar to 95ºC. Regarding the behaviour on ABTS it was the opposite with a decrease instead of an increase. TA showed the same behaviour in both assays, both lower and higher temperatures were similar and in DPPH for TL, all temperatures were similar and in ABTS all were different.

4.1.4. Correlations and dendrograms

Table VIII – Pearson's correlations (*r*) of the antioxidant assays (infusions obtained at all temperatures).

	TPC	TFC	TAA	RP	FRAP	DPPH	ABTS
TPC	1	0.596**	0.848**	0.954**	0.909**	-0.858**	-0.851**
TFC		1	0.671**	0.508**	0.456*	-0.426*	-0.430*
TAA			1	0.833**	0.777**	-0.579**	-0.582**
RP				1	0.944**	-0.764**	-0.773**
FRAP					1	-0.694**	-0.714**
DPPH						1	0.909**
ABTS							1

**, Correlation is significant at the 0.01 level. *, Correlation is significant at the 0.05 level.

TPC, total phenolic content; TFC, total flavonoid content; TAA, total antioxidant activity; RP, reducing power; FRAP, ferric reducing antioxidant power; DPPH, DPPH free radical scavenging activity; ABTS, ABTS free radical scavenging activity.

Table VIII shows the Pearson's correlations coefficients ($-1<r<1$) between the assays performed, all of them significant. The correlations obtained ranged from very strong ($|r|>0.900$) to strong ($0.7<|r|<0.9$), moderate ($0.5<|r|<0.7$) and weak ($0.3<|r|<0.5$). The correlation values between both DPPH and ABTS and the other assays are negative. This is due to the fact that higher IC_{50} values mean lower free radical scavenging activity, in contrast with the other assays where a higher value means a higher content or activity. In practice, they are inversely correlated and when the value of one variable increases the value of the other variable decreases.

Strong correlations were obtained between the total phenolic content and the rest of the assays except TFC. These results suggest that antioxidant activity is directly related with the phenolic compounds content present in the infusions and this can be used as an indicator of the antioxidant potential of a plant. The strong negative correlation between the TPC and both DPPH and ABTS indicates that the phenolic compounds have a good capacity to scavenge both free radicals. Regarding the reducing power methods, the correlations obtained showed

that the antioxidants present in the infusions have a good capacity to reduce iron. Total flavonoid content showed a moderate correlation with TPC, TAA and RP and a weak one with FRAP and both DPPH and ABTS. Some correlations are not so strong due to the presence of phytochemical compounds other than those quantified in this work which can contribute to the extracts antioxidant activity.

Since CE infusion was the least similar to the other infusions, new correlations were made with all infusions except CE (data not shown). These new correlations obtained were subtrated to Table VIII to be possible understand if this factor influence global correlations for better or worse. Almost every correlation value decreased except the free radical scavenging (DPPH and ABTS) with the other assays, which greatly improved. This result wasn't expected and it was a surprise to verify that CE was not an outlier regarding assay correlations. Then another correlation was performed to ascertain how the lower/higher temperatures could influence the assays, grouped and individually. In general when lower temperatures are grouped (25 and 50ºC) the correlations were worse in almost every assay and at the higher ones (75 and 95ºC) almost every correlation was better (except ABTS with both TFC and DPPH). The highest influence for the worst was observed at 25ºC (the correlations decreased between the assays), while at 95ºC the oposite was observed. In conclusion it was possible to understand that lower temperatures have a much bigger influence in the correlations for the worst. A possible explanation is that lower temperatures are not enough to extract certain phytochemicals that influence the antioxidant capacity of the plants in a aqueous extract.

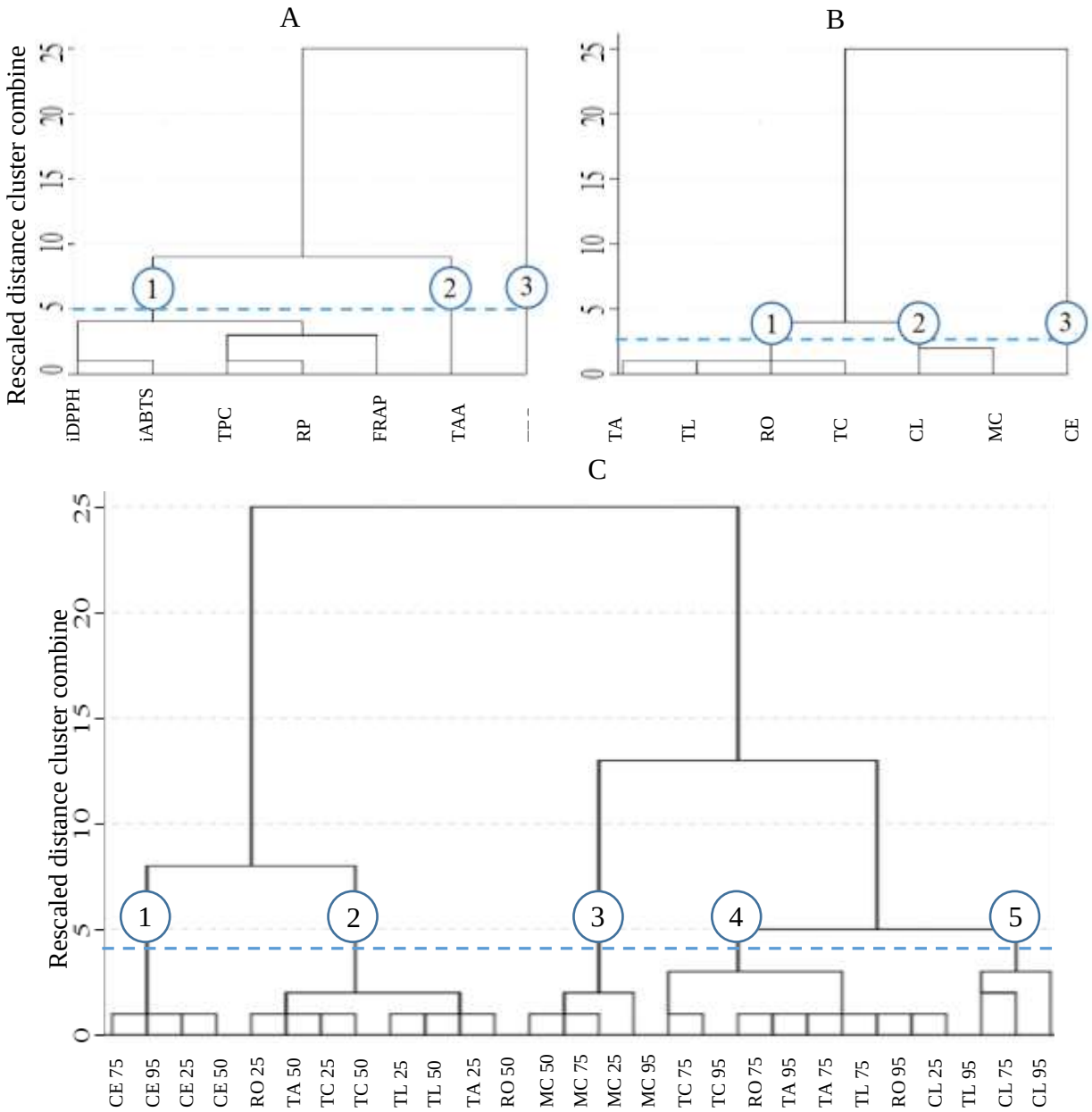


Figure 7 – Dendrograms of the global relations between assays (A), plants (B) and plants at each temperature (C).

TPC, total phenolic content, TFC, total flavonoid content; TAA, total antioxidant activity; RP, reducing power; FRAP, ferric reducing antioxidant power; iDPPH, inverse value of DPPH free radical scavenging activity; iABTS, inverse value of ABTS free radical scavenging activity. CE, *C. erythraea;* CL, *C. ladanifer*; MC, *M. communis*; RO, *R. officinalis*; TC, *T. capitata*; TA, *T. albicans*; TL, *T. lotocephalus.* The numbers 25, 50, 75 and 95 correspond to the extraction temperatures (in ºC).

Dendrograms are the graphical representation of the Euclidean distances between data points, connecting first those with the shortest distance, which means most similar data. In the assays dendrogram (figure 7 - A) the values of DPPH and ABTS had to be inverted due to the fact that higher IC_{50} values mean lower free radical scavenging activity, in contrast with the other assays. In this dendrogram, a "z-score" transformation/analysis was performed due to the range of the assays performed, with different units and scales, thus allowing the program

to relate the results of the assays by behaviour and not by closer values. This option allows more reliable results. Three diferent clusters (in each dendrogram) where identified in dendrograms A (assays) and B (plants) on figure 7. In dendrogram A, cluster 1 shows the close relationship between DPPH and ABTS (free radical scavenging activity assays) followed by TPC (phenolic content) with RP and FRAP (both ferric reducing activity), which means that these three assays are more similar between themselves than with the first two which is in accordance with the correlations, where RP is better correlated with TPC than with FRAP and these less with DPPH and ABTS. Cluster 2 contains only TAA and cluster 3 only TFC. This last one had a distinct behaviour from the other assays and for that reason is less correlated, appearing in an isolated cluster, as expected. TAA is different enough to be isolated but still close to cluster 1. The last one has the least resemblance to the other assays, which is corroborated by the lower values of correlation presented on Table VIII. In the assays performed, three infusion groups stood out for the results obtained earlier which justifies dendrogram B. Cluster 1 contains the four aromatic plants ((*Rosmarinus* - RO, both *Thymus* – TA and TL and *Thymbra* - TC)), which presented higher resemblance between them. This might be due to the fact that these aromatic plants belong to the same order despite the different genus. Cluster 2 has CL and MC which stood out due too their higher values in all assays. Lastly on the third cluster, CE appears isolated due to the lower values in the majority of the assays.

In dendrogram C (relation between plants at each temperature), 5 clusters were identified. Cluster 1 corresponds to CE, a plant with a very particular characteristic, low in antioxidant content but curiously the one with the highest flavonoid/phenolic proportion. Cluster 2 and 4 show how the four aromatic plant infusions are related at lower (25 and 50ºC) and higher extraction temperatures (75 and 95ºC). In cluster 3 all temperatures of the MC infusion are together and in cluster 5, it is possible to find three of the four temperatures of CL infusion. These results are in accordance with the hipothesis that temperature influences the extraction of phytochemical compounds, mainly in aromatic plants (*Rosmarinus* - RO, both *Thymus* – TA and TL and *Thymbra* - TC), and how CE, MC and CL are isolated due to the specificity of these plant infusions in the assays performed.

4.1.5. General conclusions

Several assays were performed in order to determine if the infusions have or not a good antioxidant activity. This is desirable because each method measures different characteristics of the antioxidant compounds which allow us to better characterize the plants antioxidant activity.

Overall the antioxidant activity average was higher for myrtle (MC) (in 5 of the 7 assays) and rockrose (CL) (in 3 of the 7 assays), proving that both plants have the potential to prevent diseases related with oxidative stress. This result for myrtle is in accordance with the bibliography where its cold and hot infusions were on the top three when compared to 9 other wild plants collected in Algarve.

Analysing all results it is possible to conclude that aromatic plant infusions (*Rosmarinus* - RO, both *Thymus* – TA and TL and *Thymbra* - TC) often had statistical similarities between them, which was expected due to the fact that all of them belong to the family Lamiaceae. These infusions were also more times statistically similar with the rest of the other plants, which suggests the presence of the same active compounds. CL infusion was more similar with MC than with any other infusion and these two were the least influenced by temperature in the extraction of phytochemical compounds with water and in some assays it even showed no effect.

In general results were grouped in the two lowest or highest temperatures, which means using 25 or 50ºC and 75 or 95ºC gives approximatly the same results. For that reason further studies on these plants using water as extraction solvent, could be done using only temperatures of 25 and 75ºC to avoid additional expenses. To obtain an infusion from the tested plants with the best antioxidant activity the temperature of 75ºC should be used. The MC infusion was overall the best at all extraction temperatures and the exceptions were TFC at the four temperatures and TAA at 50 and 75ºC, where CL was the best instead.

The majority of the plants analysed have compounds, capable of being extracted with water, with good antioxidant activity and are a rich source of phytochemical compounds, which have beneficial effects on human health, preventing oxidative stress and might be especially rockrose and myrtle (CL and MC) ,useful in the treatment of a wide range of ailments.

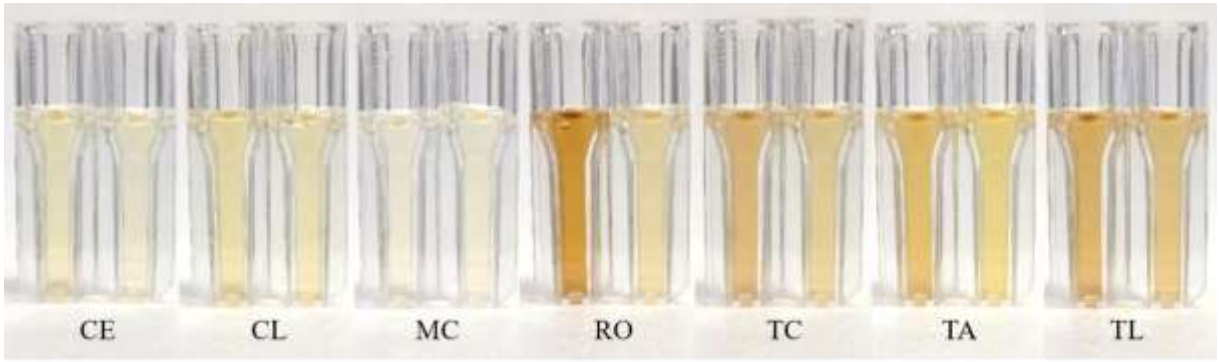
Colourimetric analysis and antidiabetic activity

4.1.6. Colourimetric assay

Another group of assays was performed, this time a colourimetric analysis to understand if the colour of the infusions is directly related with the quantity of antioxidant and posteriorly with the antidiabetic activity. Since the results obtained for the antioxidant assays were very similar between 25 and 50ºC and 75 and 95ºC for the next experiments only two of the four temperatures were used in order to be more economical. The temperature of 25ºC is considered room temperature and has the advantage of not being influenced by the heat and it is not necessary an external heat source and 75ºC was the temperature with the best results.

For the colourimetric assay, the plant infusions were diluted to the minimum concentration of the extracts, which was 0.003 g of dry weight/mL (figure 8) and a characterization was made regarding their colour intensity and yellow, red and blue proportions.

Figure 8 – Colour of the seven plant infusions (diluted to 0.003 g/mL) obtained at 25 and 75ºC, respectively.



Analysing the figure 8, two facts should be considered: all aromatic plant infusions (*Rosmarinus* - RO, both *Thymus* – TA and TL and *Thymbra* - TC) present higher colour intensity and consequently infusions with lower colour intensity presented higher antioxidant activity (except CE). The second fact is that in general, infusions at 25ºC have more colour intensity than the ones at 75ºC. As expected, the highest colour proportion of the infusions was yellow, the light is reflected by the flavonoids and yellow is the transmited colour. The yellow colour is determined at 420 nm. Red is determined at 520 nm due to the reflection of the light from betalains and finally the blue colour is determined at 620 nm due to the reflection colour of a specific subclass of flavonoids - anthocyanins. The green proportion

was not determined because the solvent used was water which is incapable of extracting chlorophyll.

Table IX – Colour of the infusions obtained at 25 and 75ºC.

		CE	CL	MC	RO	TC	TA	TL
		Plant infusions obtained at 25ºC						
Colour Intensity		0.56	1.00	0.34	2.32	1.91	2.29	1.84
Proportion (%)	Yellow	72.19	77.56	70.47	58.63	57.16	54.93	62.43
	Red	17.65	14.33	18.71	29.19	27.17	27.59	25.35
	Blue	10.16	8.12	10.82	12.18	15.67	17.48	12.21
		Plant infusions obtained at 75ºC						
Colour Intensity		0.40	0.85	0.60	1.21	1.17	1.46	1.50
Proportion (%)	Yellow	70.65	68.67	62.58	61.24	62.10	68.81	61.87
	Red	19.15	19.08	22.85	23.88	24.11	19.84	24.33
	Blue	10.20	12.25	14.57	14.88	13.80	11.35	13.80

Colour intensity was calculated as the sum of the three colour absorbances. Yellow proportion (420 nm), red (520 nm) and blue (620 nm) were also determined by the multiplication of the colour absorbances for 100 and divided by CI. CE, *C. erythraea;* CL, *C. ladanifer*; MC, *M. communis*; RO, *R. officinalis*; TC, *T. capitata*; TA, *T. albicans*; TL, *T. lotocephalus.*

Through the colour intensity, with the use of a spectrophotometer, it was possible observe that aromatic plants (*Rosmarinus* - RO, both *Thymus* – TA and TL and *Thymbra* - TC) had the highest values of colour intensity at both temperatures (Table IX). This fact is corroborated by the visual analysis of the infusions. The predominant colour was yellow, as expected. In the plant infusions obtained at 25ºC, yellow proportion values were between 54 - 77%, red between 14 – 29% and blue between 8 – 17%. At 75ºC yellow proportion values were between 61 – 70%, red 19 – 24% and blue 10 – 14%.

4.1.7. Antidiabetic activity

To determine the potential antidiabetic activity, two enzymes associated with the carbohydrates digestion which can lead to complications in type 2 diabetes patients were used. The α-amylase enzime was the first used in the determination of the tested extracts IC_{50} as a measure of their antidiabetic activity. It was only possible to obtain the IC_{50} for CL (1277.65 ± 202.81 and 1763.36 ± 183.65 µg/mL) and MC (914.76 ± 62.49 and 2663.02 ± 283.37 µg/mL) at both temperatures of 25 and 75ºC, respectively. Due to this fact, a calibration curve with the standard acarbose was made to determine the acarbose equivalents of the aqueous extracts used. The acarbose IC_{50} obtained was approximately 260 µg/mL.

Only three of the seven plant infusions tested (CE, CL and MC), showed enough activity at 25ºC (Table X) to allow the convertion of their inhibition into acarbose equivalents. It is used acarbose as a standard since it is used as a drug to treat *diabetes mellitus*. At 75ºC CL, MC, RO, TA and TL showed activity. For TC (that showed the lowest values) it was not possible to determine neither the IC_{50} or the concentration in the acarbose calibration curve at any temperature. Although TC didn't show antidiabetic activity with this enzyme, this does not mean that it does not have it. A possible reason to justify this can be an insufficient amount of extracted phytochemical compounds with α-amylase inhibitory activity, since the infusions were made with water and without stirring, another possible reason is the differences between the phytochemicals of each plants, both the profile and amount.

These results show how extraction temperature is a factor which can higly influence the potential antidiabetic activity of the tested plants (better at higher temperatures which was similar to the antioxidant activity).

The other enzyme used was α-glucosidase which was tested in the infusions extracted at 25ºC. The values obtained were much higher than α-amylase in acarbose equivalents. The α-amylase enzyme was more sensitive to acarbose than α-glucosidase (α-amylase = 0.20 mg Ac/mL versus α-glucosidase = 26.67 mg Ac/mL for a 40% inhibition), while for the extracts it was the opposite, since the concentration necessary to obtain the same inhibition was lower for α-glucosidase than for α-amylase (in α-amylase MC was diluted 9x versus a 1300x dilution for α-glucosidase for a 40% inhibition). These results show that α-glucosidase is an enzyme with potencially more afinity to the compounds present in the plant infusions than α-amylase. Since the results in α-amylase at 75ºC were better than at 25ºC and these last were higher in α-glucosidase, it is possible to assume that the values in α-glucosidase at 75ºC whould be much higher than those obtained at 25ºC.

Table X – Results of the antidiabetic activity of the seven plant infusions in acarbose equivalents determined by α-amylase (infusions at 25 and 75ºC) and α-glucosidase (infusion at 25ºC).

	T (°C)	Plants						
		CE	CL	MC	RO	TC	TA	TL
α-amylase (µg AcE/g dw)	25	192.20 ± 13.71	674.06 ± 48.61	2004.83 ± 179.74	n/q	n/q	n/q	n/q
	75	n/q	946.80 ± 79.90	868.06 ± 58.32	184.78 ± 18.81	n/q	154.09 ± 16.94	163.88 ± 13.30
α-glucosidase (mg AcE/g dw)	25	16.19 ± 1.08	> 4028.59	4984.99 ± 227.22	505.52 ± 101.34	299.07 ± 39.30	676.27 ± 11.56	705.78 ± 88.60

The results are presented as mean ± standard deviation (of the triplicates). α-amylase, α-amylase inhibition assay; α-glucosidase, α-glucosidase inhibition assay; AcE, acarbose equivalents; dw, dry weight; n/q, not quantifiable due to the low inhibitory activity.

CE, *C. erythraea;* CL, *C. ladanifer*; MC, *M. communis*; RO, *R. officinalis*; TC, *T. capitata*; TA, *T. albicans*; TL, *T. lotocephalus.*

Once again CL and MC presented the best results in comparison with all the other plants and curiously CE and MC had in α-amylase a better result at 25ºC than at 75ºC, possibly due to the degradation of certain compounds at higher temperatures. The results of CE and RO infusions were opposite to the results expected, because they have a recognized antidiabetic potential in bibliography, this shows how only one or two assays may not be suficient to conclude about any activity (antioxidant or antidiabetic) of the plant infusions.

An aspect to considerer is, due to the costs of enzimatic assays, using other protocols to do a screening of the plant infusions and relate them with the antidiabetic activity to avoid additional costs with infusions without activity, for instance.

To test infusions obtained at 75ºC for instance, the use of TAA assay may be a good option to conclude about the antidiabetic potencial of an infusion.

Correlations were made between the results of the colourimetric and antidiabetic assays obtained from the infusions at 25ºC (data not shown) and it was possible to find a significant negative correlation of -0.724 ($r<0.05$) between the colour intensity and α-amylase. On the other hand α-glucosidase is not correlated with the colour intensity. Another correlation was done using all the antioxidant, colourimetric and α-amylase inhibition assay results from the infusions obtained at 75ºC. In this case α-amylase had a positive correlation with TPC and TAA, and negative with DPPH and ABTS.

Further studies should be conducted to better characterize the antidiabetic activity demonstrated by the infusions.

4.1.8. General conclusions

The search for safer and more effective natural pharmaceuticals is desirable since the hypoglycaemic drugs have many undesirable effects.

Although small centaury (CE) is well referenced in bibliography to treat many diseases, including the ones caused by oxidative stress, it did not show good results in the antioxidant assays when compared with the other six plant infusions tested. Once again, lower temperatures can extract some phytochemical compounds which at higher temperatures are degraded although they cannot extract compounds that are extracted at higher temperatures. This means significant differences were expected and found between the infusions obtained at the highest and lowest temperatures. Despite this fact, the infusions extracted at higher temperatures, showed higher antidiabetic activity than the ones extracted at lower temperatures.

Myrtle (MC) and rockrose (CL) infusions were the most effective against both enzymes tested, similarly to the observed in the antioxidant assays. This suggests both plants could be used as potential glycaemic controlling agents helping to alleviate symptoms related to *diabetes mellitus*.

5. Final conclusion

Several assays were used to determine the phenolic content, antioxidant and antidiabetic activities in aqueous infusions of medicinal plants and two plants stood out for their results. Myrtle or MC and rockrose or CL presented the highest potential to be used in the prevention of many diseases caused by oxidative stress due to its antioxidant activity.

From all the tested plants myrtle provided the infusion with the highest antioxidant activity at all extraction temperatures, but especially at 75ºC.

These two plants also showed the highest potential to be used in the control of complex carbohydrates digestion by α-glucosidase (better than acarbose) and α-amylase. Their high α-glucosidase inhibition could be explored to complement the α-amylase inhibition of acarbose and allow a better glycaemic control.

Strong correlations were obtained between the total phenolic content and the rest of the antioxidant assays except with TFC and the HCA confirmed and showed how temperature influenced the infusions.

6. Bibliographic references

1. Šiler B, Avramov S, Banjanac T, Cvetković J, Nestorović Živković J, Patenković A, et al. Secoiridoid glycosides as a marker system in chemical variability estimation and chemotype assignment of Centaurium erythraea Rafn from the Balkan Peninsula. Industrial Crops and Products. 2012;40:336-44.
2. Kumarasamy Y, Nahar L, Cox PJ, Jaspars M, Sarker SD. Bioactivity of secoiridoid glycosides from Centaurium erythraea. Phytomedicine. 2003;10(4):344-7.
3. Valentao P, Fernandes E, Carvalho F, Andrade PB, Seabra RM, Bastos ML. Hydroxyl radical and hypochlorous acid scavenging activity of small centaury (Centaurium erythraea) infusion. A comparative study with green tea (Camellia sinensis). Phytomedicine. 2003;10(6-7):517-22.
4. Tahraoui A, Israili ZH, Lyoussi B. Acute and sub-chronic toxicity of a lyophilised aqueous extract of Centaurium erythraea in rodents. J Ethnopharmacol. 2010;132(1):48-55.
5. Radović M, Šiler B, Živković JN, Banjanac T, Živković S, Nikolić M, et al. Bioreactor Cultivation of Zeltnera beyrichii (Torr. & A. Gray) Mans.: A Novel Source of Biologically Active Compounds. Rec Nat Prod. 2013;7:4:266-80.
6. Piatczak E, Wielanek M, Wysokinska H. Liquid culture system for shoot multiplication and secoiridoid production in micropropagated plants of Centaurium erythraea Rafn. Plant Science. 2005;168(2):431-7.
7. Siler B, Zivkovic S, Banjanac T, Cvetkovic J, Nestorovic Zivkovic J, Ciric A, et al. Centauries as underestimated food additives: antioxidant and antimicrobial potential. Food Chem. 2014;147:367-76.
8. Hamza N, Berke B, Cheze C, Agli AN, Robinson P, Gin H, et al. Prevention of type 2 diabetes induced by high fat diet in the C57BL/6J mouse by two medicinal plants used in traditional treatment of diabetes in the east of Algeria. J Ethnopharmacol. 2010;128(2):513-8.
9. Loizzo MR, Saab AM, Tundis R, Menichini F, Bonesi M, Piccolo V, et al. In vitro inhibitory activities of plants used in Lebanon traditional medicine against angiotensin converting enzyme (ACE) and digestive enzymes related to diabetes. J Ethnopharmacol. 2008;119(1):109-16.
10. Sefi M, Fetoui H, Lachkar N, Tahraoui A, Lyoussi B, Boudawara T, et al. Centaurium erythrea (Gentianaceae) leaf extract alleviates streptozotocin-induced oxidative stress and beta-cell damage in rat pancreas. J Ethnopharmacol. 2011;135(2):243-50.
11. Haloui M, Louedec L, Michel J-B, Lyoussi B. Experimental diuretic effects of *Rosmarinus officinalis* and *Centaurium erythraea*. Journal of Ethnopharmacology. 2000;71:465-72.
12. Calvo MI, Cavero RY. Medicinal plants used for cardiovascular diseases in Navarra and their validation from official sources. J Ethnopharmacol. 2014;157:268-73.
13. Hamza N, Berke B, Cheze C, Marais S, Lorrain S, Abdouelfath A, et al. Effect of Centaurium erythraea Rafn, Artemisia herba-alba Asso and Trigonella foenum-graecum L. on liver fat accumulation in C57BL/6J mice with high-fat diet-induced type 2 diabetes. J Ethnopharmacol. 2015;171:4-11.
14. Valentão P, Fernandes E, Carvalho F, Andrade PB, Seabra RM, Bastos ML. Antioxidant Activity of *Centaurium erythraea* Infusion Evidenced by Its Superoxide Radical Scavenging and Xanthine Oxidase Inhibitory Activit. Journal Agric Food Chem. 2001;49:3476-79.
15. MANSAR-BENHAMZA L, DJERROU Z, HAMDI PACHA Y. Evaluation of anti-hyperglycemic activity and side effects of *Erythraea centaurium* (L.) Pers. in rats. African Journal of Biotechnology 2013;12(50):6980-85.
16. SOSA T, CHAVES N, ALIAS JC, ESCUDERO JC, HENAO F, GUTIÉRREZ-MERINO C. INHIBITION OF MOUTH SKELETAL MUSCLE RELAXATION BY

FLAVONOIDS OF *Cistus ladanifer* L.: A PLANT DEFENSE MECHANISM AGAINST HERBIVORES. Journal of Chemical Ecology. 2004;Vol. 30(6):1087-101.
17. Andrade D, Gil C, Breitenfeld L, Domingues F, Duarte AP. Bioactive extracts from Cistus ladanifer and Arbutus unedo L. Industrial Crops and Products. 2009;30(1):165-7.
18. Ramalho PS, Freitas VAPd, Macedo A, Silva G, Silva AMS. Volatile components of *Cistus ladanifer* leaves. FLAVOUR AND FRAGRANCE JOURNAL. 1999;14:300-2.
19. Papaefthimiou D, Papanikolaou A, Falara V, Givanoudi S, Kostas S, Kanellis AK. Genus Cistus: a model for exploring labdane-type diterpenes' biosynthesis and a natural source of high value products with biological, aromatic, and pharmacological properties. Front Chem. 2014;2:35.
20. Guimarães R, Barros L, Carvalho AM, Sousa MJ, Morais JS, Ferreira ICFR. Aromatic plants as a source of important phytochemicals: Vitamins, sugars and fatty acids in Cistus ladanifer, Cupressus lusitanica and Eucalyptus gunnii leaves. Industrial Crops and Products. 2009;30(3):427-30.
21. Sánchez-Vioque R, Polissiou M, Astraka K, Mozos-Pascual Mdl, Tarantilis P, Herraiz-Peñalver D, et al. Polyphenol composition and antioxidant and metal chelating activities of the solid residues from the essential oil industry. Industrial Crops and Products. 2013;49:150-9.
22. Tomas-Menor L, Morales-Soto A, Barrajon-Catalan E, Roldan-Segura C, Segura-Carretero A, Micol V. Correlation between the antibacterial activity and the composition of extracts derived from various Spanish Cistus species. Food Chem Toxicol. 2013;55:313-22.
23. Al-Anbori DKA, Al-Nimer MSM, Al-Weheb AM. Antibacterial activity of ethanolic extract of *Myrtus communis*. L leaves against salivary *Mutans streptococci* Saudi Dental Journal. 2008;20(2):82-7.
24. Bajalan I, Ghasemi Pirbalouti A. Variation in antibacterial activity and chemical compositions of essential oil from different populations of myrtle. Industrial Crops and Products. 2014;61:303-7.
25. Serio A, Chaves-López C, Martuscelli M, Mazzarrino G, Di Mattia C, Paparella A. Application of Central Composite Design to evaluate the antilisterial activity of hydro-alcohol berry extract of Myrtus communis L. LWT - Food Science and Technology. 2014;58(1):116-23.
26. Hsouna AB, Hamdi N, Miladi R, Abdelkafi S. *Myrtus communis* Essential Oil: Chemical Composition and Antimicrobial Activities against Food Spoilage Pathogens. CHEMISTRY & BIODIVERSITY 2014;11:571-80.
27. Fani MM, Kohanteb J, Araghizadeh A. Inhibitory activity of Myrtus communis oil on some clinically isolated oral pathogens. Med Princ Pract. 2014;23(4):363-8.
28. Aleksic V, Knezevic P. Antimicrobial and antioxidative activity of extracts and essential oils of Myrtus communis L. Microbiol Res. 2014;169(4):240-54.
29. Aidi Wannes W, Marzouk B. Differences between Myrtle Fruit Parts (Myrtus communisvar.italica) in Phenolics and Antioxidant Contents. Journal of Food Biochemistry. 2013:n/a-n/a.
30. Sumbul S, Ahmad MA, Asif M, Akhtar M. *Myrtus communis* Linn. □ A review Indian Journal of Natural Products and Resources 2011;2(4):395-02.
31. Dairi S, Madani K, Aoun M, Him JL, Bron P, Lauret C, et al. Antioxidative properties and ability of phenolic compounds of Myrtus communis leaves to counteract in vitro LDL and phospholipid aqueous dispersion oxidation. J Food Sci. 2014;79(7):C1260-70.
32. Moreira MR, Salvadori MG, de Almeida AA, de Sousa DP, Jordan J, Satyal P, et al. Anxiolytic-like effects and mechanism of (-)-myrtenol: a monoterpene alcohol. Neurosci Lett. 2014;579:119-24.

33. Feisst C, Franke L, Appendino G, Werz O. Identification of molecular targets of the oligomeric nonprenylated acylphloroglucinols from Myrtus communis and their implication as anti-inflammatory compounds. J Pharmacol Exp Ther. 2005;315(1):389-96.
34. Ozkol H, Tuluce Y, Dilsiz N, Koyuncu I. Therapeutic potential of some plant extracts used in Turkish traditional medicine on streptozocin-induced type 1 diabetes mellitus in rats. J Membr Biol. 2013;246(1):47-55.
35. Celiktas OY, Kocabas EEH, Bedir E, Sukan FV, Ozek T, Baser KHC. Antimicrobial activities of methanol extracts and essential oils of Rosmarinus officinalis, depending on location and seasonal variations. Food Chemistry. 2007;100(2):553-9.
36. Ribeiro-Santos R, Carvalho-Costa D, Cavaleiro C, Costa HS, Albuquerque TG, Castilho MC, et al. A novel insight on an ancient aromatic plant: The rosemary (Rosmarinus officinalis L.). Trends in Food Science & Technology. 2015;45(2):355-68.
37. Muñoz K, Calderón J, Osorio E, Castro D, Serna R, Díaz J, et al. Rapid HPTLC-based method for quality control: simultaneous chemical analysis and antioxidant activity determination in herbal, nutraceutical and functional foods. Procedia Food Science. 2011;1:960-4.
38. Johnson JJ. Carnosol: a promising anti-cancer and anti-inflammatory agent. Cancer Lett. 2011;305(1):1-7.
39. Erkan N, Ayranci G, Ayranci E. Antioxidant activities of rosemary (Rosmarinus Officinalis L.) extract, blackseed (Nigella sativa L.) essential oil, carnosic acid, rosmarinic acid and sesamol. Food Chem. 2008;110(1):76-82.
40. Bhatt K, Kothiyal P. REVIEW ON ANTI-CHOLINESTERASE ACTIVITY OF SOME NATIONAL AND INTERNATIONAL PLANTS International Journal of Universal Pharmacy and Bio Sciences 2013;2(5):621-35.
41. Cui L, Kim MO, Seo JH, Kim IS, Kim NY, Lee SH, et al. Abietane diterpenoids of Rosmarinus officinalis and their diacylglycerol acyltransferase-inhibitory activity. Food Chemistry. 2012;132(4):1775-80.
42. Yun YS, Noda S, Shigemori G, Kuriyama R, Takahashi S, Umemura M, et al. Phenolic diterpenes from rosemary suppress cAMP responsiveness of gluconeogenic gene promoters. Phytother Res. 2013;27(6):906-10.
43. Bouayad N, Rharrabe K, Ghailani NN, Jbilou R, Castañera P, Ortego F. Insecticidal effects of Moroccan plant extracts on development, energy reserves and enzymatic activities of Plodia interpunctella. Spanish Journal of Agricultural Research. 2013;11(1):189.
44. Habtemariam S. The Therapeutic Potential of Rosemary (Rosmarinus officinalis) Diterpenes for Alzheimer's Disease. Evid Based Complement Alternat Med. 2016;2016:2680409.